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MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVI CYCLE

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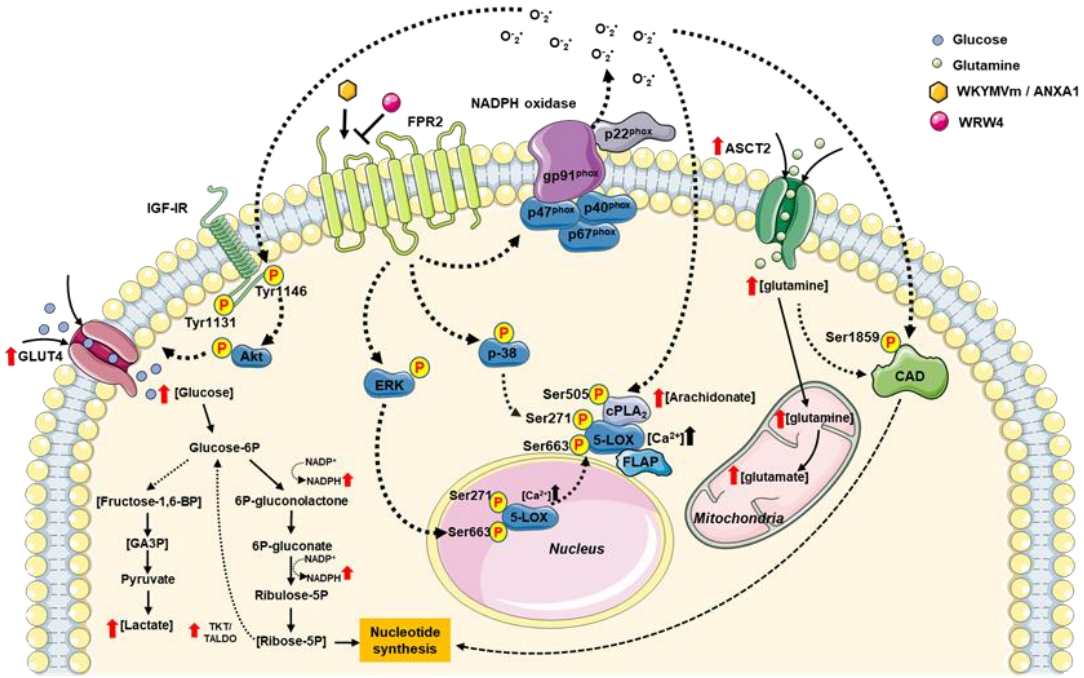
UNRAVELING THE ROLE OF FPR2 IN THE METABOLISM OF LUNG CANCER CELL



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UNRAVELING THE ROLE OF FPR2 IN THE METABOLISM OF LUNG CANCER CELL

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LIST OF ABBREVIATIONS

ATP=Adenosine triphosphate

TCA= Tricarboxyl Acid Cycle

TME= Tumor Microenvironment

ROS= Reactive Oxygen Species

OXPHOS= Oxidative Phosphorylation

ANXA1=Annexin A1

LXA4= Lipoxin A4

SAA= Serum amyloid A

HN=Humanin

c-Met= Hepatocyte Growth Factor Receptor

HSP-27= Heat Shock Protein-27

MCM2= Mini-Chromosome Maintenance protein 2

Rb= Retinoblastoma

OSR1= Oxidative-Stress- Responsive kinase 1

MARCKS= Myristolated Alanine-Rich C-Kinase Substrate

IRI= Ischemia riperfusion injury

RvD1= Resolvin D1

PFK= Phosphofructokinase

PDK1= Pyruvate Dehydrogenase Kinase 1

LDH-A= Lactate Dehydrogenase A

ALDOA= Aldolase A

PGK1= Phosphoglycerate Kinase 1

PK= Pyruvate Kinase

MCT= Monocarboxylate transporter

PDAC= Pancreatic Ductal Adenocarcinoma

α- KG= α- Ketoglutarate

ASCT2= Sodium-dependent neutral amino acid transporter type 2

GLS= Glutaminase

GLUD= Glutamate Dehydrogenase

GOT= Glutamate Oxalacetate Transaminase

PPAT= Phosphoribosyl Pyrophosphate Amidotransferase

FASN= Fatty Acid Synthase

SCD1= Stearoyl-CoA Desaturase 1

FATPs= Fatty Acid Transporter Protein

SCARB1= Scavenger B Receptor type 1

LIMP-2= Lysosomal integral membrane protein

CPT1= Carnitine Palmitoyl Transferase 1

CT2= Carnitine Transporter

PKM2= M2 isoform of Pyruvate Kinase

GIRK= G-protein-coupled inwardly rectifying potassium channels

G6P= Glucose-6-Phosphate

F1,6BP= Fructose 1,6-Bisphosphate

GA3P= Glyceraldheide-3-Phosphate

ECAR= Extracellular Acidification Rate

OCR= Oxygen Consumption Rate

PER= Proton Efflux Rate

R5P= Ribose 5-P

ASP=Aspartate

GLU= Glutamate

GLN= Glutamine

PPP= Pentose Phosphate Pathway

G6PDH= Glucose-6-P-Dehydrogenase

AMP= Adenosine monophosphate

CMP= Cytidine monophosphate

UMP= Uracil monophosphate

THF= N10-formyl-Tetra-Hydrofolate

TKT= Transketolase

TALDO= Transaldolase

dUDP= Uridine diphosphate

dCDP= Cytidine diphosphate

CAD= Carbomoyl-phosphate synthetase 2, aspartate transcarbamylase, dihydroorotase

AA= Arachidonic Acid

cPLA2= Phospholipase A2

LOX= Lipoxygenase

COX= Cyclooxygenase

LT= Leukotrienes

Sommario

1. ABSTRACT

Background: Formyl peptide receptors (FPRs) belong to the GPCR super-family and include three members differentially expressed (FPR1, FPR2 and FPR3) that recognize distinct ligands. FPR2 is considered the most promiscuous isoform of the family, since it can bind a wide range of synthetic or endogenous agonists, such as the peptide WKYMVm and Annexin A1 (ANXA1).

By phosphoproteomic analysis, we identified several phosphoproteins uniquely phosphorylated in FPR2-stimulated CaLu-6 cells. Interestingly, we pointed out that the largest part of these phosphoproteins is involved in the regulation of energetic metabolism. Hence, we investigated the role of FPR2 in cellular metabolism.

Results: We observed that WKYMVm or ANXA1 stimulation significantly inhibits Pyruvate Dehydrogenase activity by triggering PI3K/Akt-dependent PFKBP2 phosphorylation, thus directing glucose towards glycolysis. In addition, the concentration of several metabolites associated with the pentose phosphate pathway (PPP), tricarboxyl acid cycle, nucleotide synthesis, glutamine metabolism and lipid metabolism, was also significantly enhanced in FPR2-stimulated cells. Our results highlight that the binding of specific FPR2 agonists in lung cancer cells: (i) promotes NADPH production; (ii) activates the non-oxidative phase of PPP; (iii) induces the expression of ASCT2 glutamine transporter; (iv) regulates oxidative phosphorylation; (v) induces NOX-dependent de novo synthesis of pyrimidine; (vi) influences lipid metabolism.

Conclusions: Collectively, these data demonstrate that FPR2 is involved in metabolic reprogramming of lung cancer cells, since its stimulation modulates glucose and glutamine metabolism, regulate mitochondrial oxidative phosphorylation and promotes biosynthetic pathways, necessary for the survival of cancer cells. Therefore, FPR2 can be considered a promising therapeutic target for novel pharmaceutical therapies to treat human tumors.

2. Introduction

2.1 Cellular metabolism and metabolic reprogramming

Cancer is a genetic illness caused by dynamic alterations in the genome. Examples of changes in a cell that steer transformation toward a malignant phenotype include gain-of-function mutations that activate oncogenes, loss-of-function mutations that inactivate suppressor genes, and mutations that inactivate stability genes (Romero-Garcia et al., 2011).

Normal cells enter the cell cycle only when instructed by a finely tuned process that is dependent on neighboring cells, chemical cues in the microenvironment, and the cell itself. An external stimulus, such as growth factors, interacts with the appropriate receptor in normal cells to stimulate an intracellular signaling cascade, which can lead to cellular duplication. Genetic abnormalities in cancer cells can increase the number of growth factor receptors and intracellular signaling pathways that promote cell proliferation may be turned on or boosted on a continuous basis, allowing malignant cells to self-stimulate growth and proliferation (Romero-Garcia et al., 2011).

Energy is obtained by cells through a sequence of chemical reactions known as metabolism that consists of two different phases: anabolism and catabolism. During anabolism cells synthetize complex molecules such as proteins, lipids and carbohydrates and in catabolism these macromolecules are degraded in smaller molecules to obtain energy in the form of ATP (Deberardinis et al., 2008). Carbohydrates are transformed to glucose, which is then converted in two molecules of pyruvate. Pyruvate dehydrogenase complex catalyzes the formation of Acetyl-coenzyme A (CoA), which can enters in the tricarboxylic acid cycle (TCA) amphibolic cycle. Citrate, oxaloacetate and succinyl-coenzyme A, TCA intermediates, can be subtracted from the cycle and represent building blocks for synthesis of lipids, aspartate, and other key macromolecules (Schiliro & Firestein, 2021). Proteins are converted into amino acids which are transamined into corresponding a-chetoacids thus feeding TCA.

Cancer cells have developed a novel yet uncommon strategy to collect energy from the outside in order to sustain their uncontrolled growth pace. Normal cells, in presence of oxygen, do not convert glucose to lactate and only use anaerobic glycolysis to convert glucose to lactic acid, when oxygen is scarce or unavailable. Cancer cells, on the other hand, convert glucose to lactate even in presence of oxygen (aerobic glycolysis) by a process called ***Warburg effect***. This phenomenon was identified approximately 100 years ago by Otto Warburg who proposed that cancer cells have changed metabolism as a consequence of a mitochondrial damage determining a permanent impairment of oxidative metabolism (Warburg O., 1956). However, the involvement of mitochondria in tumor cells has long been debated since many tumor cell lines with high

proliferative rates do not show oxidative metabolic abnormalities (Moreno-Sánchez et al., 2007). Mitochondria play crucial roles in cellular activities such as ATP production, the generation of reactive oxygen species (ROS), apoptosis, and the synthesis of metabolites that serve as building blocks for biomolecules. Therefore, impaired mitochondrial function may support tumor formation by reducing apoptosis, promoting ROS generation, or activating a hypoxia-like pathway under normoxic conditions. However, this theory did not explain how tumor growth could be supported under the changing conditions of the tumor microenvironment with a permanent mitochondrial impairment (Robey & Hay, 2009). Nevertheless, in the last years, it is well established that Warburg effect is not due to mitochondrial impairment, but, instead, cancer cells use this phenomenon for generating several glycolytic intermediates that sustain anabolic pathways, such as pentose phosphate pathway, important for NADPH and ribose-5-phosphate production, amino acid, lipids, and other cellular sources of energy (Hamanaka & Chandel, 2012) (Lunt & Vander Heiden, 2011).

Metabolic reprogramming is one of the hallmarks of cancer cells and is adopted to sustain their rapid growth and proliferation by ensuring a sufficient supply of proteins, nucleotides, and lipids (Ward & Thompson, 2012). The most well-known altered metabolic processes in cancer cells are glycolysis and glucose metabolism, but, more recently, also other metabolic pathways are observed to be involved in metabolic reprogramming. In particular, it has been observed that cancer cells use glutamine for growth and proliferation (Wang et al., 2020). Glutamine in cancer cells plays different roles providing not only nitrogen for amino acid and nucleotide biosynthesis but it is also a source of carbon to restore TCA and lipid biosynthesis pathway, in this way cancer cells are "addicted" to glutamine (Altman et al., 2016b). Furthermore, more recently also lipid metabolism it has been observed crucially involved in cancer development. Lipids, together with proteins and nucleic acids, are critical components of biological membranes. They are also key signaling molecules for numerous cellular activities and are involved in energy storage and metabolism. Therefore, cancer cells use lipids to get energy, membrane components, and generates signaling molecules required for proliferation, survival, inflammation, invasion, metastasis, and response to tumor microenvironment effect and cancer therapy (Bian et al., 2021).

Inflammation is frequently linked to the onset and progression of cancer. In fact, cancer cells, as well as surrounding stromal and inflammatory cells, generate an inflammatory tumor microenvironment (TME) through well-coordinated reciprocal interactions. TME cells are highly flexible, constantly changing their phenotypic and functional properties (Greten & Grivennikov, 2019). In particular, chronic inflammation is associated with different steps of tumorigenesis, including cellular transformation, survival, proliferation,

invasion, angiogenesis, and metastasis (Singh N. et all, 2019). The role of inflammation in cancer development is related to both intrinsic and extrinsic pathways. In the intrinsic pathway, genetic alterations that cause neoplasia trigger the expression of inflammation-related programs which, in turn, guide the formation of an inflammatory microenvironment, whereas in extrinsic pathway, inflammatory conditions contribute to cancer development (Aggarwal et al., 2009). Inflammation is characterized by reactive oxygen species (ROS) generation. ROS are a class of chemically reactive compounds in which the superoxide anion ($^{\bullet}O_2^-$) serves as the primary precursor to other species, such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}HO$) (Weyemi et al., 2013). While mitochondria constitute a major source of ROS as a byproduct, NOX/DUOX of NADPH oxidase family appears to be unique since it produces ROS in a physiological manner. Polymorphonuclear neutrophils (PMNs), that express NADPH oxidase, produce ROS involved in the host- defense response. In several cell types ROS can act both as mediator of inflammation and signaling molecules (Mittal et al., 2014). Notably, it has been observed that malignant transformation is linked to an increased ROS generation and there are also mounting evidences that members of the NOX/DUOX family have roles in cancer biogenesis and or development (Hsieh et al., 2011).

2.2 Glucose metabolism in cancer cells

In the 1920s Otto Warburg demonstrated that tumor ascites showed a high glucose consumption rate and lactate production, despite having enough oxygen available to completely oxidize glucose. This phenomenon is called Warburg effect (Romero-Garcia et al., 2011). According to the Warburg effect, cancer cells rely on aerobic glycolysis (the conversion of glucose to lactate in the presence of oxygen) for ATP synthesis, whereas normal cells rely on oxidative phosphorylation (OXPHOS) (Ward & Thompson, 2012). The molecular mechanisms that support the Warburg effect involve several critical molecular players.

Glucose metabolism represents one of the most significant aspects of cancer cells since it provides intermediates and precursors for several other key metabolic pathways, including the production of amino acids, nucleotides, and lipids (Vander Heiden et al., 2009) (**Figure 1**).

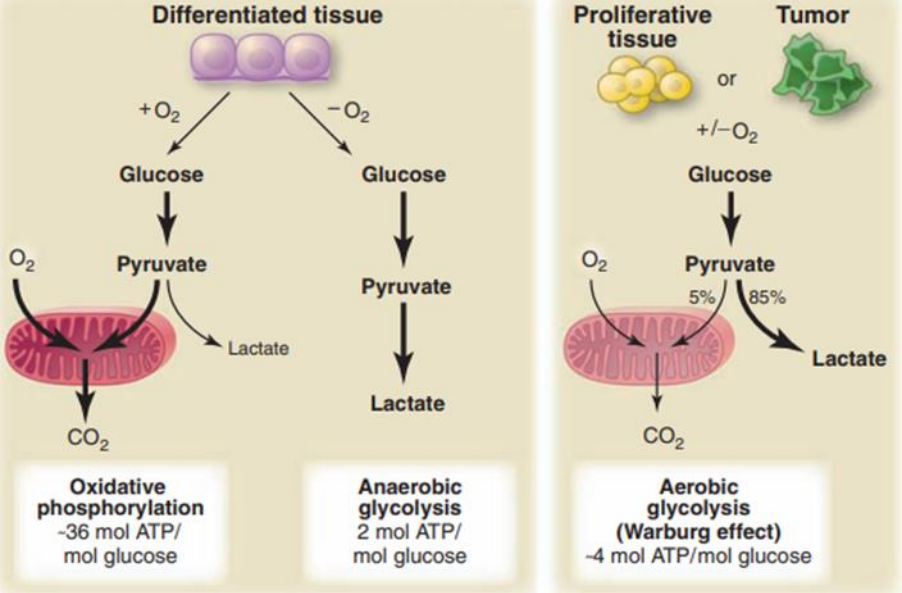

Figure 1. Differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg effect).

Non-proliferating (differentiated) tissues convert glucose to pyruvate via glycolysis and then totally oxidize most of that pyruvate in the mitochondria to CO2 via the process of oxidative phosphorylation in the presence of oxygen. When oxygen is scarce, cells can divert glycolysis-generated pyruvate away from mitochondrial oxidative phosphorylation by producing lactate (anaerobic glycolysis). In anaerobic glycolysis, lactate is useful to continue glycolysis, as it restores NAD^+, but this process results in a reduction of ATP production if compared to oxidative phosphorylation. Warburg discovered that cancer cells convert the majority of glucose to lactate in spite of oxygen is present (aerobic glycolysis). However, aerobic glycolysis produces less ATP than oxidative phosphorylation.

Adopted from ***Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science, 324(5930), 1029-1033.***

There is an important difference between OXPHOS and aerobic glycolysis in terms of ATP production. OXPHOS generates 36 ATPs per mole of glucose, whereas glycolysis produces 2 ATPs for one glucose. However, lactate generation is faster than OXPHOS and since cancer cells have to sustain the proliferation rate, they prefer to obtain less energy in as little time as possible (Läsche et al., 2020). The characteristic uncontrolled cell growth of cancer cells requires to create energy quickly and this is achieved via aerobic glycolysis. The increased lactate production also contribute to an acidic microenvironment wherein only cells with acid-resistance characteristics can proliferate. In this environment, cancer cells get a significant growth edge and became more invasive and metastatic (Ghanavat et al., 2021). Hypoxia is a common feature of solid tumors, and it is promoted mainly by rapid cell proliferation and inadequate vascularization. Metabolic reprogramming is influenced by hypoxia and in this tumor microenvironment cells use certain stress mechanisms to survive (Kreuzaler et al., 2020). In particular, in this stress response, oncogenes and tumor suppressors activate specific molecules and signaling pathways involved in aerobic glycolysis, such as proto-oncogene Myc, transcription factor hypoxia inducible factor 1 (HIF-1), the PI3K/Akt/ mTOR pathway, and tumor suppressor p53, which are known to be involved in different types of cancer (Schiliro & Firestein, 2021). In particular, Myc is a transcriptional factor that regulates the expression of many genes involved in glycolysis, such as glucose transporters (GLUTs), the glycolytic enzymes hexokinase 2 (HK2), phosphofructokinase (PFK), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase (PDK1). The PI3K/Akt/mTOR pathway improves the efficiency of glycolytic enzymes HK2 and PFK, by Akt signaling (Park et al., 2020). Therefore, Myc signaling and PI3K/Akt pathway result upregulated in cancer cells. The p53 tumor suppressor serves as a stress sensor and is a key regulator of cellular activities, such as cell cycle arrest, apoptosis, DNA repair, metabolic reprogramming, stemness, invasion and migration (Ozaki & Nakagawara, 2011). p53 is mutated into several types of cancer impairing glycolysis by downregulating GLUT1, GLUT4 and HK2 (Itahana & Itahana, 2018).

Another important protein involved in the regulation of glucose metabolism is HIF-1 (Moldogazieva et al., 2020). HIF-1 is an important transcriptional factor, sensing oxygen concentration. At high oxygen concentration, the subunit HIF-1α is unstable and rapidly degraded, whereas it is activated by low oxygen concentration. In its activated form, HIF-1 is an heterodimer that consists of two subunits: HIF-1α, the regulatory subunit expressed depending on oxygen concentration and HIF-1β which is costitutively expressed. In the nucleus this heterodimer binds to hypoxia-response enhancer sequence, hypoxia-response element (HRE), to promote the expression of numerous hypoxia-responsive genes such as HK2, phosphofructokinase 1 (PFK1), aldolase A (ALDOA), phosphoglycerate kinase 1 (PGK1), pyruvate kinase (PK), and lactate dehydrogenase 1 (LDHA) (Läsche et al., 2020). On the other hand, it downregulates pyruvate dehydrogenase (PDH) activity by upregulating PDH

kinases (PDKs), preventing the entry of pyruvate into the TCA (Lu et al., 2015). HIF-1 also promote the expression of glucose transporters to increase glucose uptake and enhances lactate secretion by inducing the expression of monocarboxylate transporters (MCTs) (Nagao et al., 2019). HIF-1 induces glycolysis and prevents OXPHOS by inhibiting Complex I activity (Tello et al., 2011).

The definition of ***"metabolic plasticity"*** was recently adopted to describe the property of cancer cells to retain fully functional OXPHOS machinery and at the same time to switch between OXPHOS and aerobic glycolysis, or even do both at the same time (Moldogazieva et al., 2020). This new theory could fully explain the ability of cancer cells to adapt to the different microenvironment and to avoid cell death. As mentioned before, the first theory on metabolic reprogramming formulated by Warburg suggested that cancer cells prefer glycolysis since mitochondria activity was impaired, whereas the new concept of plasticity overturned the Warburg theory (Warburg O., 1956). In fact, many reports show an upregulation of OXPHOS in multiple types of cancer, such as melanoma (Kumar et al., 2021), pancreatic ductal adenocarcinoma (PDAC) (Nie et al., 2020), leukemia and subset of lymphomas (Ashton et al., 2018). This process highlights the relevance of interaction and molecular communication in cancer cell metabolism and shows that elevation of aerobic glycolysis is not a hard and fast rule in the tumor microenvironment.

2.3 Glutamine metabolism in cancer cells

Glutamine is an essential amino acid that provides a second important source of energy for cancer cells and its increased consumption in cancer growth is widely recognized as a hallmark of the disease (Altman et al., 2016a). Moreover, it has been observed that glutamine depletion induces apoptosis of malignant cells (Yuneva et al., 2007) and this dependence on glutamine metabolism is known as ***"glutamine addiction"*** . Glutamine plays a key role in cell viability since it participates in several transduction pathways (Cacace et al., 2017) and it is used for proteins, lipids and nucleotides synthesis. Glutamine is also the precursor for the biosynthesis of glutamate (Glu), which once converted into α-ketoglutarate (α-KG), enters into the TCA (J. Zhang et al., 2017). In particular, cancer cells enhance glutaminolysis to replenish TCA cycle and to supply lipid production, amino acid synthesis and the pentose phosphate pathway (Kalyanaraman, 2017), depending on tumor, patient and cancer type heterogeneity (Cioce et al., 2020). Furthermore, glutamine is useful also for glutathione biosynthesis (Yu et al., 1999). Glutaminolysis converts glutamine into glutamate which, in turn, drives the production of energy via lactate (Erickson & Cerione, 2010). By avoiding OXPHOS and the formation of ROS, this process further supplies cancer cells required for their rapid growth and proliferation. Glutamine's metabolism is regulated by

different oncogenes (Yang et al., 2017). Among these, the proto-oncogene c-Myc binds to the promoter regions of glutamine importers, such as ASCT2 (sodium-dependent neutral amino acid transporter type 2, also known as SLC1A5) and SN2 (isoform of system N, also known as SLC38A5), enhancing glutamine uptake (Wise et al., 2008). Furthermore, c-Myc overexpression increases mitochondrial glutaminase (GLS) expression via transcriptional suppression of miR-23a and miR-23b (Gao et al., 2009). c-Myc also directly targets genes involved in dNTP (deoxyribonucleotide triphosphate) metabolism in order to improve glutaminolysis and generate amide nitrogens required for nucleotide synthesis (Mannava et al., 2008). Oncogenic KRAS elevates the expression of enzymes involved in glutamine metabolism, in particular it downregulates expression of glutamate dehydrogenase (GLUD) and upregulates the expression of glutamate oxaloacelate transaminase (GOT). The oxaloacetate is converted into malate and then pyruvate, increasing the $NADPH/NADP^+$ ratio into cytoplasm (Son et al., 2013). PI3K/Akt/mTOR pathway is downregulated in many cancers and it is also involved in glutamine metabolism. Specifically, this axis can also activate nuclear factor-like 2 (NRF2), a redox-sensitive transcription factor that regulates a variety of processes, including glutathione (GSH) synthesis and regeneration (Wang et al., 2008). Glutamine metabolism may also be controlled by tumor suppressors. In fact, p53 triggers the expression of glutaminase isozyme (GLS2) which has tumor suppressor effects, and it eliminates ROS to protect cells against oxidative stress (Suzuki et al., 2010). Others tumor suppressors involved in glutamine metabolism are retinoblastoma (Rb) and liver kinase 1 (LKB1), which alter glutamine uptake by inhibiting the expression of ASCT2 by E2F transcription factor 3 (E2F3) (Reynolds et al., 2014) (Faubert et al., 2014). Other genes implicated in glutamine metabolism include IDH1/2 (Borodovsky et al., 2012), glutamate dehydrogenase (GDH) (Ni et al., 2023), pyruvate carboxylase (PC) (Cheng et al., 2011), phosphatidylinositide 3-kinase (PI3K), signal tranducer and activator of transcription 1 (STAT1) (Zhao et al., 2013) (Zhao et al., 2012), extracellular signal-regulated kinases (ERKs) (Thangavelu et al., 2012). Recently, a ***"second Warburg-like effect"*** in glutamine nitrogen metabolism has been described (Kodama et al., 2020) and it could explain the cancer cell metabolism shift from glutaminolysis to de novo nucleotide production. This metabolic reprogramming is under the control of GLS1 and of the rate-limiting enzyme phosphoribosyl pyrophosphate amidotransferase (PPAT) (Kodama et al., 2020). Recently it has been suggested that glutaminolysis may inhibit nucleotide byosinthesis and hamper cancer cell growth since redirecting glutamine nitrogen metabolism could not be advantageous for cancer cells. However, it has been observed that cancer cells can compensate for the loss of glutamine-derived carbon sources caused by this metabolic change by consuming glucose. Although further studies are required to corroborate that Warburg-like effect on glutamine metabolism could be as significant as the original Warburg effect on carbon metabolism.

2.4 Lipid metabolism in cancer cells

Lipid metabolism reprogramming is considered a further crucial metabolic alteration observed in cancer cells and it significantly contributes to cells growth and tumorigenesis (Bian et al., 2021). The family of lipid includes sterols, mono/di/triglycerides, phospholipids, and glycolipids and they are indispensable for the cells being energy sources, components of biological membranes and signaling molecules (Koundouros & Poulogiannis, 2020). In particular, phospholipases (PLC, PLD and PLA) can produce several second messengers including diacylglycerol, phosphatidic acid, lysophosphatidic acid and arachidonic acid.These molecules induce the activation of RAS, PI3K, protein kinase C, RAC,RHO, and a number of other signaling pathways that can promote cancer (Moolenaar & Perrakis, 2011) (Park et al., 2012). Considering the numerous functions exerted by lipids, impairment of the mechanisms regulating their amounts are crucially involved in cancer progression. In fact, several elements of lipid metabolism are reprogrammed in cancer, including fatty acid (FA) biosynthesis and oxidation, FA uptake from the environment and FA modification and release from other molecules (Bian et al., 2021). In cancer cells it was observed an increased reliance on de novo biosynthesis and exogenous FA absorption to sustain their fast proliferation rate and supply energy demand during metabolic stress conditions (Röhrig & Schulze, 2016). FAs in mammalian cells, can be obtained either by direct exogenous uptake from the surrounding microenvironment or through de novo synthesis using nutrients such as glucose or glutamine. It is commonly known that lipidomic remodelling is a metabolic signature of cancer which includes changes in FA transport, de novo lipogenesis, storage as lipid droplets (LDs), and oxidation to generate ATP (Monaco, 2017). Cells can receive lipids through either de novo synthesis or absorption (Cheng et al., 2018). The majority of lipids are produced from the long carbon chains of FAs. In adult cells, FAs are generally generated from external sources such as diet or lipids produced by liver, whereas in cancer cells is preferred de novo lipogenesis in order to eliminate dependency on external sourced lipids and to proliferate more rapidly (Koundouros & Poulogiannis, 2020). FA is synthetized via cytoplasmic acetyl-CoA, which is produced from acetate, glucose, or glutamine. The enzymes acetyl-CoA carboxylases (ACC1/2) and fatty acid synthase (FASN) convert this acetyl-CoA to malonyl-CoA and finally to palmitate. Palmitate is subsequently extended to other FAs or desaturated by FA elongases and FA desaturases to form the cellular pool of non-essential FAs, which are eventually transformed to other key lipids like cholesterol, eicosanoids, and prostaglandins. In many cancers, it has been observed an increase of FA de novo synthesis, which is essential for cancer growth (Currie et al., 2013) (Guo et al., 2013). In cancer cells de novo synthesis is promoted by upregulating many enzymes involved in this pathway, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), and stearoyl-CoA desaturase 1 (SCD1) (Cheng et al., 2018). In particular, PI3K/ Akt/ mTORC1 signaling enhances the expression of enzymes required for FA synthesis

and in turn promotes de novo lipogenesis. Furthermore, PI3K-mediated NRF2 activation can contribute to increase the production of NADPH, which is employed as a cofactor in FA synthesis processes (Koundouros & Poulogiannis, 2020). The enhanced de novo lipogenesis contribute to the malignant phenotype of cancer cells. In particular, cancer cells acquire the ability to shunt into different biosynthetic pathways and to produce a wide variety of lipids with different functions that assure them to proliferate. Furthermore, FA synthesis protects cancer cells from ROS, promotes pro-angiogenic signaling, and provides an escape from immune surveillance (Röhrig & Schulze, 2016). However, in the hypoxic condition that characterized the tumor microenvironment, it has been hypothesized that malignant cells on one hand reduce FA synthesis and on the other hand increase uptake of exogenous lipids (Kamphorst et al., 2013). Lipid uptake can take place via a variety of mechanisms, including the use of specialized transporters such as the CD36 fatty acid translocase or the fatty acid transport proteins (FATPs of the SLC27 family of solute carriers), or receptor-mediated endocytosis of low-density lipoprotein (LDL) particles via the LDL receptor (LDLR). To note, all these mechanisms are highly expressed in various types of cancer (Schiliro & Firestein, 2021) and it has been also demonstrated that FA uptake favors tumor migration and metastasis (Röhrig & Schulze, 2016). HIF-1 is considered the master regulator of exogenous lipid uptake since it controls the expression of lipid-binding proteins, such as FA- binding protein 4 (FABP4) (Koundouros & Poulogiannis, 2020).

A different process used by tumor to obtain fatty acids is the lipolysis. Lipolysis consists of a breakdown of lipid droplets by lipoprotein lipase (LPL) to release free FAs (Maan et al., 2018). Protein CD36 transports these free FAs into the cells where they support an increased growth. CD36 is a membrane protein in the class B scavenger receptor family, which also contains scavenger B receptor type 1 (SCARB1) and lysosomal integral membrane protein (LIMP-2) (Park, 2014). It is expressed on the surface of several cells, such as adipocytes, monocytes, macrophages, platelets, cardiomyocytes, dendritic cells, epithelial cells, erythrocytes and muscle cells. In malignant cells, it alters lipid metabolism by increasing fatty acid absorption, it enhances proliferation and promotes epithelial-mesenchymal transition (Guerrero-Rodríguez et al., 2022). In several types of cancer, it has been observed an increased expression of CD36 and LPL, suggesting that tumors use this process to improve the availability of FAs as well as for growth and proliferation (Kuemmerle et al., 2011) (Prieto et al., 2018).

Free fatty acids can be broken down further by oxidation (FAO), also known as β-oxidation. β-oxidation contributes to produce FADH2, NADH and ATP to obtain the energy required for biosynthetic processes. New evidence supports the idea that FAO plays an important role in cancer growth and metastasis than what was thought before (Ma et al., 2018). Accordingly, it has been demonstrated an increased expression of many FAO enzymes in cancer, for example CD36, rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1), carnitine transporter CT2 (Zhao et

al., 2013) , and Acyl-CoA synthetase long chain 3 (Kuemmerle et al., 2011) (Ma et al., 2018). This effect is due to an overexpression of oncogenic c-Myc (Ma et al., 2018). FAO has also been linked to metastasis due to its putative function in the reprogramming of cancer stem cells (C. Wang et al., 2019). These data support the idea that FAO plays an essential role in cancer metabolism.

2.5 NADPH oxidase in cancer cells

The NOX family is the primary source of controlled ROS production, and it consists of seven isoform with diverse tissue distribution and activation mechanisms (Schröder, 2020). In particular, NOXs subcellular distribution varies depending on cell types, spanning from the plasma membrane to intracellular compartments and the nuclear membrane (Moloney et al., 2017). NOX1, NOX2, NOX3, NOX4, NOX5, and the dual oxidase DUOX1 and DUOX2 are all members of the NOX family. NOX2 was the first identified isoform, and it is composed of at least six distinct subunits which must interact in order to create an active enzyme complex (Pecchillo Cimmino et al., 2023). In particular, NOX2 consists of two integral membrane proteins gp91phox and p22phox (cyt b558), and three cytosolic regulatory subunits p40phox, p47phox and p67phox, and Rac1/2. In unstimulated cells, NOX results inactivated and its subunits are not associated with each other (Touyz et al., 2002) whereas upon stimulation p47phox is phosphorylated on several serine residues and this event promotes its interaction with p67phox. This interaction induces the translocation on the membrane of all cytosolic subunits where they bind the cyt b558 and in turn trigger NOX activation. Once activated, NOX complex is able to produce superoxide anion. Through a prosthetic group (flavin) and a heme group(s), the activated NOX transfers electrons from the substrate (NADPH) to molecular oxygen (**Figure 2**). Except for NOX4 which is constitutively active (F. Chen et al., 2012), other members of NOX family are strictly regulated in order to avoid ROS overproduction (Edens et al., 2001; Lambeth et al., 2004). NOX-dependent superoxide generation is important in phagocytic leukocytes for destroying phagocytosed organisms and facilitating cell anti-microbial function (Nauseef, 2019), whereas in the most of cells and tissues NOX-dependent ROS production is involved in biosignaling (Begum et al., 2022) and pathophysiological functions, including cardiovascular (Caso et al., 2021; Sylvester et al., 2022), neurodegenerative (Ganguly et al., 2021), cancer (Szanto, 2022) and metabolic diseases (Elumalai et al., 2021; Nascè et al., 2022). As signaling molecules, ROS trigger the covalent modification of specific cysteine residues found inside redox-sensitive target proteins. As a result of this oxidation the activity of protein tyrosine phosphatases (PTPs), and of several other enzymes is reversibly altered (Finkel, 2011), promoting the phosphorylation of cytosolic residues of tyrosine kinase receptors (TKRs) (Castaldo et al., 2019) (Cattaneo et al., 2013b) and serine/threonine receptors (RSTK) (Mohamed et al., 2019) (Cattaneo et

al., 2014). In many types of cancer cell lines and human tumors at early and late stages of tumorigenesis, it has been described an increased expression of NOX1, NOX2, NOX4 and NOX5, implying that they are involved in cancer development and progression (Meitzler et al., 2014) (Höll et al., 2016). It has also been shown that the loss of DUOX1 expression in lung cancer cell lines is strongly associated with the loss of the epithelial marker E-cadherin, and that DUOX1 silencing promotes the features of an epithelial-to-mesenchymal transition (EMT), which is an important feature of metastatic cancer (Little et al., 2016). NOX1 was the first homolog of NOX2, expressed in different tissues (Bedard & Krause, 2007), but it is most prevalent in colon, prostate, and vascular cells (Pecchillo Cimmino et al., 2023). Genomic instability NOX1 has been linked to regulation of p53 activity. NOX1 expression is regulated by K-RAS, a proto-oncogene that has a key role in tumor growth. K-RAS enhances NOX1 expression through RAF/MEK/ERK-dependent phosphorylation of the transcription factor GATA6 (Adachi et al., 2008). When upregulated NOX1 acts as an oncogene, elevating glycolysis and providing additional NAD^+ in cancer cells with mitochondrial dysfunction (Faria & Fortunato, 2020). Moreover, it also plays an important role in glutamine metabolism, since it inversely correlates with mitochondrial glutamate dehydrogenase and positively correlates with aspartate aminotransferase. Decreased NOX1 levels result in decreased cytosolic hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) expression. Since HMG-CoA synthase is involved in lipid, steroid (including cholesterol), and isoprenoid biosynthesis, NOX1 may contribute to lipid metabolism in tumor cells (Bertram et al., 2015). NOX2 is mainly expressed in monocytes, macrophages, and granulocytes where it contributes to innate immune response (Pecchillo Cimmino et al., 2023). However, its expression has also been found in nonphagocytic cells, including neurons, hepatocytes, hematopoietic stem cells, endothelial cells, cardiomyocytes, and skeletal muscle myocytes (Pecchillo Cimmino et al., 2023). Enhanced NOX2-derived ROS production creates an oxidative microenvironment that characterizes tumorigenesis, tumor progression, cell proliferation (Grauers Wiktorin et al., 2020) and cell metabolism (Griffiths et al., 2017). Tumor microenvironment is characterized by hypoxia and this induces the activation of HIF-1. NOX2-dependent ROS generation induces the activation of HIF-1 (Grauers Wiktorin et al., 2020). It has been observed that in ovarian cancer cells, NOX2-dependent HIF1α activation triggers glycolysis by enhancing the expression of GLUT1, a glucose transporter, and glycolytic enzyme hexokinase 2 (HK2) (Ha et al., 2018) (Lee et al., 2014). In primary acute myeloid leukemia (AML) cells, NOX2 is the main source of superoxide. In these cells, NOX2-dependent ROS production stimulates a rise in the uncoupling protein 2 (UCP2) and AMPK phosphorylation, hence increasing the expression of 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase (PFKFB3), a crucial regulatory glycolytic enzyme. PFKFB3 overexpression enhances glucose absorption and cell proliferation, whereas its downregulation strongly reduces leukemia growth both in vitro and in vivo (Robinson et al., 2020). Moreover, in this tumor NOX2-derived

ROS are also involved in sphingolipid metabolism, fatty acid oxidation (FAO), purine metabolism and amino acid balance. In fact, the blocking NOX2 functions alter sphingosine and sphinganine levels, as well as FAO transport, purine metabolism and amino acid balance (Robinson et al., 2021). NOX4 is considered the most evolutionary distant NOX homolog with only 39% similarity with NOX2. It has four splice variants and it has been described in several tissues and cell types (Siques et al., 2021) (Demircan et al., 2022) (Larson-Casey et al., 2021) (Eid et al., 2022; Ryu et al., 2019). It is localized in mitochondria (Shanmugasundaram et al., 2017), endoplasmic reticulum (ER) (Sciarretta et al., 2013), focal adhesion (Hilenski et al., 2004), nuclei (Guida et al., 2014) and it is linked to actin network (Vukelic et al., 2018). NOX4-derived ROS regulate a number of cellular activities in various tissues and cell types, including cell proliferation, migration, survival, transformation, cell metabolism and metabolic reprogramming. NOX4 directs glucose toward glycolysis and PPP for the production of NADPH in non-small cell lung cancer (NSCLC) cells. It also promotes glutamine metabolism for GSH synthesis via ROS/PI3K/Akt signaling, contributing to these cells' oxidative adaptation (Zhang et al., 2014). In glioblastoma, NOX4 is involved in HIF-1 stabilization through ROS generation. In particular, NOX4 overexpression enhances aerobic glycolysis, whereas its knockdown significantly reduces aerobic glycolysis (Su et al., 2021). NOX4-dependent HIF-1 stabilization has also been found in human neuroblastoma, SH-SY5Y cells, where NOX4 expression is increased in hypoxic conditions. NOX4 knockdown prevents hypoxia-induced glycolysis by inhibiting HIF activation, glycolysis-related protein expression, such as LDHA and PDK1, glucose uptake, lactate generation and ROS production (Yu et al., 2019) (29426376). Interestingly, in cancer cells without a functional NOX4, the expression of HIF1- targeting genes, such as SLC2A1, which encodes a glucose transporter, is suppressed, emphasizing the relevant role of NOX4 in metabolic reprogramming (Yu et al., 2019). NOX4 is also observed in renal carcinoma cells, where it localizes to the inner mitochondrial membrane in these cells and it is allosterically regulated by ATP levels, contributing to metabolic reprogramming (Shanmugasundaram et al., 2017). In particular, NOX4 enhances PKM2 expression, suggesting that it plays the key role of mitochondrial energetic sensor and fulfils the function of metabolic checkpoint, coupling the metabolic switch to cancer cell survival (Shanmugasundaram et al., 2017).

NOX-dependent ROS generation plays a significant role in the development of several malignancies, pathologies and disease (Esposito & Carsana, 2019; Lambeth, 2007). Interestingly, in some cancers such as neuroblastoma, epithelial, endothelial, fibroblast and lung cancer it has been observed that NADPH oxidase dependent ROS-generation are involved in intracellular signaling cascades (Castaldo et al., 2019) (Cattaneo et al., 2018) (Cattaneo et al., 2013b). In nonphagocytic cells, binding of various ligands to their respective GPCR, including angiotensin II (Nguyen Dinh Cat et al., 2013), urotensin-II (Diebold et al., 2012), endothelin-1

(Montezano & Touyz, 2012), thrombin (Diebold et al., 2009), extracellular ATP (Díaz-Vegas et al., 2015), and adenosine (Zhou et al., 2015), enhances NADPH oxidase-dependent ROS production. The first observation that a GPCR can activate NADPH oxidase was made in neutrophils, where the binding of N-formylmethionyl-leucyl-phenilalanine (N-fMLP) to formyl peptide receptor 1 (FPR1), a GPCR member, resulted in phosphorylation of NADPH oxidase cytosolic subunits, which translocated on membrane and formed an enzymatic active complex (Chessa et al., 2010; Raad et al., 2009). Cancer cells are characterized by high level of oxidative stress. This condition is due to an altered balance between production and accumulation of ROS in cells and tissue and the ability of biological systems to eliminate these products. ROS are a class of chemically reactive compounds in which the superoxide anion ($\bullet O_2^-$) serves as the primary precursor to other species such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet HO$). They are mainly produced as a natural byproducts of cellular respiration, although they can also be the result of the interaction of ionizing radiation with water molecules (Finkel, 2011). ROS are responsible for a variety of tasks, including apoptosis, homeostasis, wound healing, immunity and cell signaling (Lambeth, 2007). Interestingly, ROS exert a dual function since, at high concentration, they induce cytotoxicity, whereas at low concentration they act as second messengers (Panieri & Santoro, 2016). It was shown that in anaplastic lung cancer cells, CaLu-6 cells, the stimulation of FPR2, an isoform of FPRs family, with synthetic agonist WKYMVm peptide or with an endogenous pro-resolving ligand ANXA1, induces the phosphorylation of HSP-27, OSR1 and MARCKS as well as the activation of p38MAPK, PI3K and PKCδ, the upstream kinases of HSP-27, OSR1 and MARCKS, respectively. NADPH-oxidase dependent ROS generation are important players in the signaling cascade initiated by the two pro-resolving FPR2 agonists. In fact, the blockade of NADPH oxidase functions prevented HSP-27, OSR1 and MARCKS phosphorylation as well as the activation of their upstream kinases, suggesting that ROS are crucially involved in intracellular signaling cascades of malignant cells (Ammendola et al., 2021).

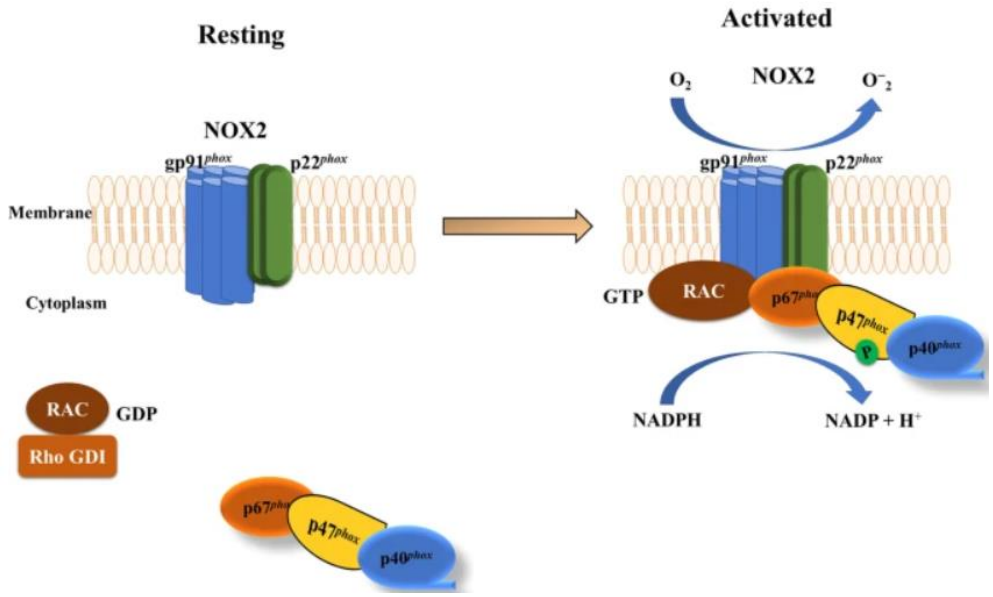

Figure 2. NADPH oxidase activation.

The classical NADPH oxidase consists of three cytosolic proteins (p47phox, p40phox and p67phox), Rac, and two membrane subunits (gp91phox, also known as NOX2 and p22phox). In resting cells these subunits don't interact with each other and NADPH oxidase is in inactive state. Whereas when NADPH oxidase is active, cytosolic subunits translocate to the plasma membrane where they interact with membrane subunits. After electrons are transported from NADPH to molecular oxygen via FAD and hemes, this multisubunit enzyme creates O2.

Adopted from ***Begum, R.; S., Abdulkadir; A. et all. NADPH oxidase family proteins: signaling dynamic to disease management. Cell Mol Immunol 19, 660-686 (2022).***

2.5.1 The role of NADPH oxidase in metabolic reprogramming

Several evidences suggest that NOX-dependent ROS generation crucially contribute to regulate cell metabolism by modulating glucose, lipid, nucleotide and protein metabolism, as well as promoting metabolic reprogramming of cancer cells (**Figure 3**). The effect of ROS on cancer biology are pleiotropic and complex.

In general, their action on tumor is determined by (1) their mutagenic potential, which is required for tumor initiation, (2) their effects on intracellular signaling pathways controlling cell proliferation and survival (Burdon et al., 1990) (3) their impact on cell motility and invasiveness (Pani et al., 2010) and (4) their recognized role in stromal reactivity, which is required for cancer development and dissemination, such as inflammation, tissue repair, de novo angiogenesis and malignancy (Toullec et al., 2010). In malignant cells, an increased concentration of ROS can be due to an enhance basal metabolic activity, mitochondrial dysfunction, peroxisome activity, uncontrolled growth factor of cytokine signaling, oncogenic activity or an enhanced NADPH oxidase activity (Fiaschi & Chiarugi, 2012). Furthermore, in different types of cancer, including breast, prostate, and colon carcinoma, as well as melanoma, it has been clearly established that oxidative stress players are abnormally expressed in cancers and contribute to metabolic reprogramming. Malignant cells are known to undergo Warburg metabolic reprogramming, which is characterized by increased aerobic glycolysis activity and lipid metabolism dysregulation. This metabolic switch occurs during cancer formation and affects metabolic flux and network topology of pathways in central carbon metabolism. In particular, oxidative stress leads to limitation of oxidative phosphorylation (Pavlides et al., 2012) and to cysteine oxidation as well as inactivation of the M2 isoform of pyruvate kinase (PKM2), resulting in an increase in the amount of glycolytic intermediates that are reconverted to the pentose phosphate pathway. This adaptation was caused by an accumulation of phosphoenolpyruvate, due to ROS-dependent inhibition of PKM2. Phosphoenolpyruvate activates pentose phosphate pathway by inhibiting glycolytic enzyme triosephosphate isomerase, and so it enhances oxidative metabolism and prevents ROS accumulation. In addition, NADPH is also used for fatty acids biosynthesis (Anastasiou et al., 2011) (Hitosugi et al., 2009), another metabolic pathway altered in cancer cells.

To note, the most observed metabolic consequences of NOX-generated ROS are related to glucose metabolism.

Glucose and glutamine play a key role in the metabolic reprogramming of cancer cells and are the principal carbon atom sources for the production of several molecules. Glutamine, in particular, is a key nitrogen donor for the synthesis of nucleotides, amino acids, and nicotinamide. In glucose metabolism, ROS can contribute to glucose uptake. In fact, the exogenous addition of hydrogen peroxide stimulates glucose uptake in skeletal muscle (Higaki et al., 2008) (Jensen et al., 2008) (Kim et al., 2006), C2C12 myoblast and 3T3 fibroblasts. Cellular glucose uptake is mediated by glucose transporters (GLUTs), which include fourteen isoforms with distinct kinetic

characteristics and different modes of regulation as well as tissue-specific expression (Carruthers et al., 2009). For example, GLUT1 is highly expressed during all stages of embryonic development. Although its expression diminishes after birth, most cell types continue to express the low amount of GLUT1 to mediate basal glucose uptake. GLUT1 expression, on the other hand, remains high in cells that predominantly rely on glycolysis for ATP synthesis, such as erythrocytes and tumor cells. GLUT1 is commonly up-regulated in the latter, which is associated with poor survival in a variety of malignant tumors (Liemburg-Apers et al., 2015). GLUT4, instead, is up-regulated in differentiating muscle cells, which have larger amounts of OXPHOS complexes and higher respiration rates (Remels et al., 2010).

ROS regulate GLUTs expression through HIF-1 activation. In fact, the GLUT1 protein expression is regulated by a promoter region and numerous putative enhancer regions, which contain binding sites for several transcription factors such as HIF1 (Pragallapati & Manyam, 2019).

Hypoxic inducible factor 1 α (HIF-1α) plays a key role in glucose metabolic reprogramming (Semenza, 2003). In fact, as a transcriptional factor, it triggers the transcription of several genes that encode proteins involved in angiogenesis, glucose metabolism, cell proliferation/survival, and invasion/metastasis. Moreover, it has been suggested that NOX-derived ROS contribute to metabolic reprogramming by stabilizing HIF-1 (Movafagh et al., 2015). In fact, ROS scavenging or NOX inhibition, totally reverses hypoxia-induced HIF-1 accumulation and hexokinase activity, supporting the hypothesis that ROS generation is upstream of HIF signaling.

During hypoxia, ROS levels rise and stabilize HIF-1 (Brunelle et al., 2005) (Chandel et al., 2000) (Guzy et al., 2005). It has been observed that in Lewis lung carcinoma, HT-29 colon, and T47D breast cancer cells, GLUT1 expression and glucose absorption are suppressed by ROS-mediated HIF1 stabilization (Jung et al., 2013). GLUT4 translocation to the plasma membrane during exercise, muscle contraction is associated with increased ROS level, which in turn stimulates glucose uptake via a mechanism associated to activation of Akt and/or AMPK (Higaki et al., 2008; Sandström et al., 2006; Toyoda et al., 2004). ROS have been shown to activate Akt (Fernandes et al., 2011), which most likely includes the inactivation of cysteine-based phosphatases. ROS have the ability to activate AMPK by modifying cysteine residues on its catalytical subunit (Zmijewski et al., 2010). Once phosphorylated, Akt and AMPK induce GLUT4 translocation (Thong et al., 2007).

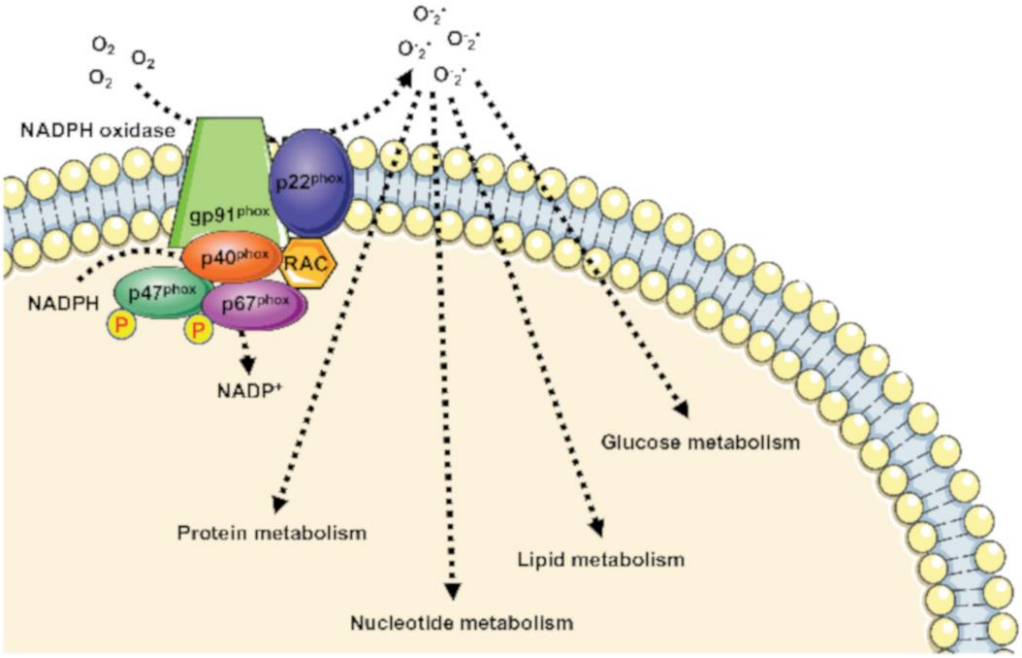

Figure 3. The role of NADPH oxidase-dependent ROS generation on cancer metabolism
Adopted from ***Pecchillo Cimmino, T.;Ammendola, R.; Cattaneo, F.; Esposito, G.; NOX Dependent ROS Generation and Cell Metabolism . Int. J. Mol. Sci. 2023,24, 2023,24,2086.***

2.6 Structure and role of GPCR

G-protein-coupled receptors (GPCRs) are versatile seven-transmembrane-domain proteins able to recognize a wide range of external signaling molecules and transduce their signals across the membrane to activate intracellular responses. They are the most numerous class of cell surface receptors and since they are involved in a plethora of physiological functions, they represent important therapeutic targets for several human disorders. In particular, they are responsible for hormonal signal transduction, sense of vision, taste and olfaction, cell survival, proliferation, and differentiation (Hall et al., 1999) (Pierce et al., 2002) (Ritter & Hall, 2009).

The GPCR structure consists of transmembrane helical domain (TMD) that spans the cell membrane, with the main ligand binding pocket on the extracellular side and a highly dynamic intracellular cleft to accommodate signaling partners. GPCRs bind a variety of extracellular chemical and physical signals, such as proteins, nucleotides, amines, peptides, ions and photons. In response to such signals, GPCRs undergo conformational changes in the TMD, allowing the recruitment of a heterotrimeric G protein and in turn to promote the G protein-mediated signaling pathways. A current classification of GPCR family is known as the A-F system, which is mostly based on amino acid sequences and functional similarities (designed fingerprints of the seven distinctive GPCR hydrophobic domains).This approach recognizes six classes of GPCR sequences from both vertebrates and invertebrates, labeled A-F. Class A, commonly known as the "rhodopsin-like family", is the biggest group of GPCRs, comprising hormones, neurotransmitters, and light receptors, and accounts for approximately 80% of all GPCRs. Class A GPCRs have seven TM helices, as well as an eighth helix and a palmitoylated cysteine at the C terminal tail. Class B, also known as the "secretin receptor family", has approximately 70 receptors with seven TM helices and a lengthy N-terminal domain of approximately 120 resides supported by disulfide bonds. The metabotropic glutamate family, GABA receptors, calcium-sensing receptors and taste receptors are all members of class C. This class is characterized by seven TM helices and a large extracellular N-terminal domain with roughly 600 ligand-binding residues. A cysteine-rich loop connects this clam-shaped domain to the TM helix 1. Class D comprises fungal mating pheromone receptors, whereas cAMP receptors are classified as class E, and frizzled/smoothened receptors are classified as class F. The amino acid sequences of GPCR in classes D-F also include seven hydrophobic domains known as TM helices. Another GPCR classification system, called "GRAFS", has been suggested based on the phylogenetic tree of about 800 human GPCR sequences. Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S) are the five major families in this system. The key distinction between GRAFS system and the A-F system is that the GRAFS system further divides class B into the Secretin family and the Adhesion family based on preliminary finding that the evolutionary histories of these two families differ (Hu et al., 2017).

A well-known method to nominate G proteins is based on their α subunits. Hence, the Gs heterotrimeric complex has Gαs; Gq has Gαq; the Gi heterotrimeric complex contains Gαi. There are four subfamilies for the Gα-subunit: Gs proteins couple to stimulation of adenylyl cyclase; Gi proteins couple to inhibition of adenylyl cyclase and the activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels; Gq proteins is linked to PLCβ activation; G_{12} proteins is associated to the activation of Rho guanine-nucleotide exchange factors (GEFs) (Pierce et al., 2002). In the inactive state, Gα is linked to Gβγ dimer and GDP. G protein-mediated signaling begins with the binding of an agonist molecule, which results in GPCR activation. In particular, in the active state GPCR can act as a guanine nucleotide exchange factors (GEFs) for heterotrimeric G protein subunits, catalyzing GDP release and GTP binding for G protein activation. The activated G protein subunits (α GTP and βγ) can subsequently interact with different downstream effectors and, in turn, to affect many aspects of cellular function (Ritter & Hall, 2009). Activated G proteins amplified GPCR signaling. Due to Gα and Gβγ proteins, which in turn attach to various effectors, switching it on or off in diverse system, and effectors continue to convey the signal to various type of second messangers. Here, intrinsic GTPase activity of Gα comes into action, resulting in the conversion of bound GTP into GDP, and, as a result, the deactivation of the G protein cascade. GTPase activity of G subunits may also be regulated by G protein signaling regulators (RGS proteins) and effectors, furthermore, effectors enzymes like as adenylyl cyclases may modulate G protein activation by receptors (Tuteja, 2009).

Activated GPCRs in its intracellular domain can be phosphorylated by also associate with a GPCR kinases (GRKs). GRKs are a group of seven related kinases (GRK1-GRK7) with distinct distribution patterns across tissues and preferences for binding to specific receptors (Moore et al., 2007; Premont & Gainetdinov, 2007). GRK-mediated GPCR phosphorylation results in a reduced GPCR connections with G proteins and an increased GPCR contacts with arrestins (member of a family of four closely related scaffold proteins). Arrestin-GPCR interaction activates arrestin-mediated recruitment of signaling proteins. In particular, arrestin signaling contribute the receptor internalization and desensitization by connecting active receptors to clathrin-coated pits and so enhancing their endocytosis (Hanyaloglu & von Zastrow, 2008) (**Figure 4**).

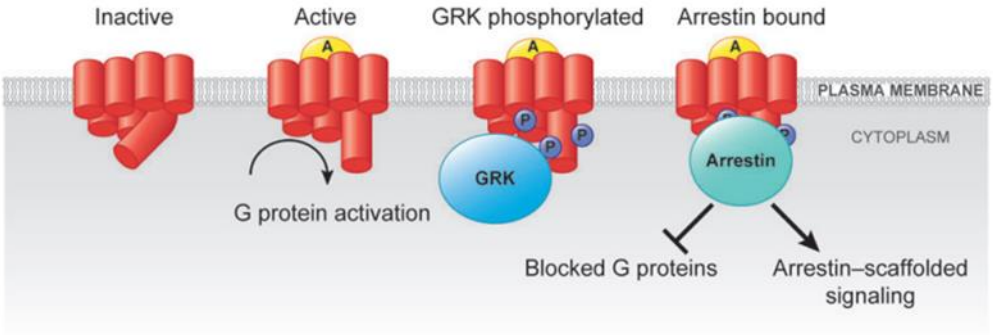

Figure 4. Activation and desensitization of GPCR

In the absence of a specific ligand (A), G-protein coupled receptor (GPCR) is in inactive form on the cell surface and it is not able to trigger any signaling pathways. When an appropriate agonist binds to the receptor at the extracellular domain, the receptor changes its conformation to exposed surfaces that act as a guanine nucleotide exchange factor for heterotrimeric G protein. In this conformation, the receptor induces the release of GDP from an inactive G- protein bound to the receptor and favors the binding of GTP to this G-protein. The GTP-bound G protein is now activated, and so it is released from the receptor, it dissociates into α and βγ-subunits, which activate downstream signals. The activate state of the receptor is also recognized by a G protein-coupled receptor kinase (GRK), which is catalytically activated by this contact. Activated GRKs phosphorylate (P) the receptor's intracellular domains before being released. The agonist-activated, GRK-phosphorylated receptor binds tightly to an arrestin protein, which prevents further G protein activation and couples the receptor to clathrin- coated pit internalization and arrestin-scaffolded (and G protein-indipendent signaling pathways).

Adopted from ***Premont, R.T. , & Gainetdinov,R.R. (2007). Physiological roles of G protein-coupled receptor kinases and arrestins. Annual review of physiology , 69,511-534.***

2.6.1 GPCR-mediated RTKs transactivation

GPCRs, in addition to canonical intracellular responses, integrates their signaling cascades by transactivating Tyrosine Kinase Receptors (RTKs) (Cattaneo et al., 2014). GPCR-mediated RTK transactivation promotes the connection of the vast diversity of GPCRs ligands with the robust signaling capacity of RTKs. Transactivation was firstly described in Rat-1 fibroblasts, where it was observed that stimulation with a variety of GPCR agonists resulted in rapid tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) (Daub et al., 1996). Furthermore, several evidences suggest that RTKs use a G protein to activate signaling pathways, and that different GPCR agonists can transactivate also members of the cell surface transforming growth factor (TGF)-receptors superfamily, which have predominantly Serine/Threonine kinase (S/TK) activity (Kamato et al., 2015), as well as Toll-like receptors (TLRs).

Transactivation can occur mainly through ligand-dependent and ligand-independent mechanisms. In the ligand-dependent triple-membrane-passing-signal (TMPS) mechanism, GPCR-mediated RTK transactivation depends on activation of membrane-bound matrix metalloproteases (MMPs), such as the A Disintegrin And Metalloprotease (ADAM) family members, (**Figure 5**). (Prenzel et al., 1999). In the ligand-independent mechanism, GPCR stimulation induces several second messengers, including Ca^{2+} ions, protein kinase C (PKC), the non-receptor protein tyrosine kinases Src and Pyk, arrestin and also ROS, which in turn induce tyrosine phosphorylation and subsequent activation of RTKs (**Figure 6**) (Esposito et al., 2011), (Gesty-Palmer et al., 2013), (El-Shewy et al., 2011), (J. Chen et al., 2012), (Tilley et al., 2009). Several molecular processes allow GPCRs to activate Src family kinases which in turn controls GPCR responses. To note, Src family tyrosine kinases plays a key role in signals transduction pathways involved in cellular growth and malignant transformation.

c-Src is the prototypic member of a family of non-receptor tyrosine kinases, that controls cell proliferation, survival, adhesion, cytoskeletal reorganization, and vesicle trafficking via cell surface receptors. Src family consists of nine members that include Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and Yrk. They have a conserved domain structure that consists of SH3, SH2, and tyrosine kinase (SH1) domains in succession (Playford & Schaller, 2004) (Reynolds & Roczniak-Ferguson, 2004). Src family kinases activation and function are regulated via their recruitment to cell surface receptors. Protein-protein interactions involving the phosphotyrosine-binding Src homology domains 2 (SH2) and proline-rich domain binding SH3 domains shared by all members of the family are used to target Src family kinases (Boggon & Eck, 2004). Src family members, for complete catalytic activity, require phosphorylation of the kinase domain. In Src this phosphorylation site corresponds to Tyr416.

In many cases, Src family kinases interact directly with cytoplasmatic receptor domains that contain consensus SH3 domain-binding motifs or proline-rich motifs within their third intracellular loops or C-terminal tails, or by binding to GPCR-associated proteins such as heterotrimeric G-protein subunits or β-arrestins. Therefore, Src family kinases

can be also activated by GPCR (Luttrell & Luttrell, 2004). Interestingly, ROS-mediated RTKs transactivation can be mediated by: (1) activating kinases as consequence of protein-protein interactions alteration; (2) inactivating protein tyrosine phosphatases by oxidizing the cysteine residues in the catalytic domain, resulting in tyrosine kinase activation; and (3) promoting proteolysis of regulatory proteins, inhibiting tyrosine kinase activity (Cattaneo et al., 2014).

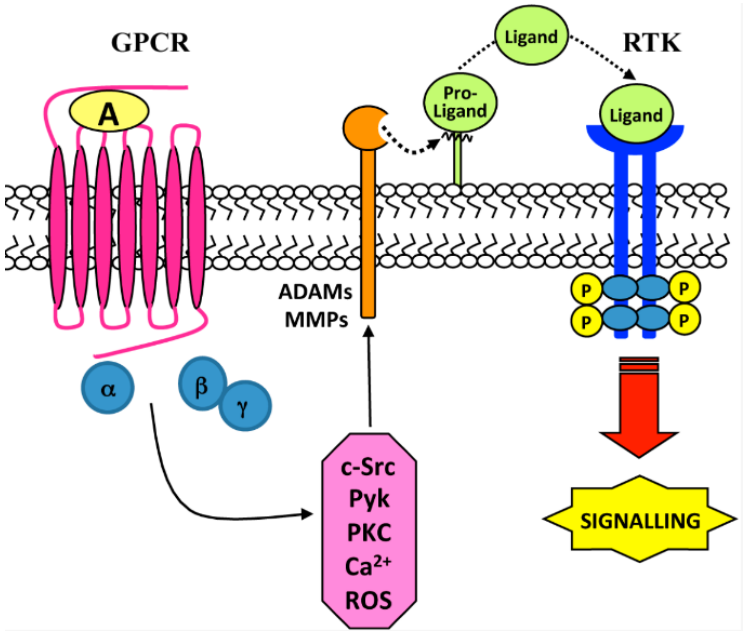

Figure 5. GPCR-mediated RTKs transactivation in a ligand-dependent manner
G-protein-coupled receptor (GPCR) stimulation with a specific agonist (A) activates several intracellular signaling mediators via activation of Gα and/or Gβγ subunits. Metalloproteases-mediated proteolytic cleavage of a Pro-Ligand results in the formation of a ligand that binds and transactivates RTK.
Adopted from ***Cattaneo, F., Guerra, G., Parisi, M., De Marinis, M., Tafuri, D., Cinelli, M., & Ammendola, R. (2014). Cell-surface receptors transactivation mediated by g protein-coupled receptors. Int J Mol Sci, 15(11), 19700-19728.***

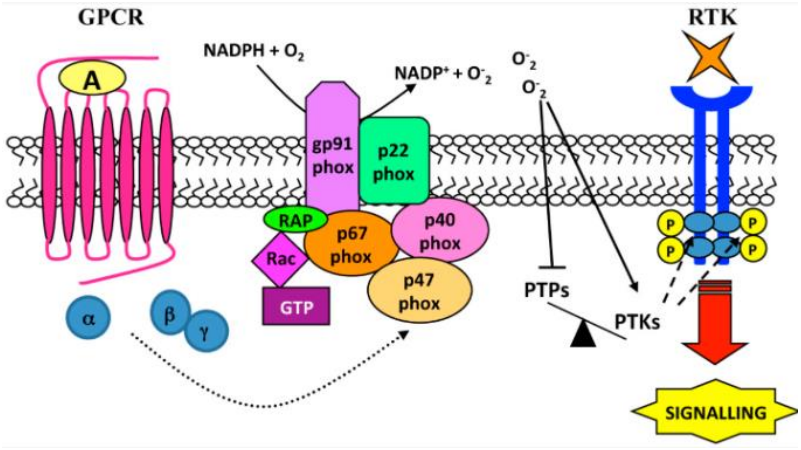

Figure 6. ROS-dependent RTK transactivation
Agonist (A) stimulation of GPCR causes p47phox phosphorylation and NADPH oxidase activation, which results in the production of ROS that act as second messengers. In particular, they inactivate protein phophatases by inducing the reversible oxidation of cysteins in the catalytic domain, unbalancing intracellular phosphorylation equilibrium. This results in an increase of phosphotyrosine.kinases (PTKs) activity that promotes the trans-phosphorylation of tyrosines in the cytosolic region of RTK, which provide docking sites for signaling complex formation and activation.
Adopted from ***Cattaneo, F., Guerra, G., Parisi, M., De Marinis, M., Tafuri, D., Cinelli, M., & Ammendola, R. (2014). Cell-surface receptors transactivation mediated by g protein-coupled receptors. Int J Mol Sci, 15(11), 19700-19728.***

2.7 Formyl peptide receptors (FPRs)

Formyl peptide receptors (FPRs) belong to the G-protein coupled receptors (GPCR) superfamily, sensitive to pertussis toxin (Chen et al., 2017). This family includes three different members in human: FPR1, FPR2 and FPR3, encoded by three genes clustered on chromosome 19q13.3-19q13.4 (Ye et al., 2009). Whereas, the mouse genome encodes seven different subtypes (referred to as Fpr) that have both overlapping and distinct roles to their human counterparts (Ye et al., 2009). Based on sequence homology, Fpr1 is regarded the equivalent of FPR1 (77% similarity), although two mouse genes, Fpr2 and Fpr3, are partial orthologues to human FPR2 due to significant sequence identity and response to lipoxin A4 (Ye et al., 2009). They were first identified and described as pattern recognition receptors that recognize peptides containing N-terminal formyl methionine generated from invading pathogens or host mitochondria as pathogen-or damage-associated chemical patterns (PAMPs and DAMPs) (He & Ye, 2017) (Zhang et al., 2010). FPR1 was the first discovered isoform of the family, in 1976, as a high affinity binding site on the surface of neutrophils for the prototic N-formyl peptide formyl-methionine-leucyl-phenylalanine (fMLP). In 1990, it was cloned from a differentiated HL-60 myeloid leukemia-cell cDNA library (Le, Y., et al., 2002). FPR1 gene is 6 kb long and has an intronless open reading frame that is interrupted by an intron in its 5'-untranslated region. It encodes for a protein of 350aa that consists of two putative N-linked glycosylation residues at position 4 and 10, and a disulphide bond at

residues 98-176. It is also characterized by three putative phosphoserine sites (residues 328, 332, and 338) and four putative phospho-threonine sites (residues 329, 331, 334, 336 and 339). Moreover, FPR1 has several alpha and beta helices, as well as seven transmembrane domains (Zhu et al., 2022). FPR2 and FPR3 were subsequently identified by low-stringency hybridization using FPR1 cDNA as a probe (Bao, L., et al, 1992) (Ye, R.D., et al,1992). FPR2 gene encodes for a protein of 351aa with one putative N-linked glycosylation at position 4 and a disulphide bond at residues 98-176. It also possesses multiple alpha helices and beta strands and seven transmembrane domains (Zhu et al., 2022). Hence, FPR1 and FPR2 are very similar in their structure. However, FPR2 shows a lower affinity for formylated peptides compared to FPR1. In fact, it has been observed that high concentrations of fMLP could induce only Ca^{2+} mobilization but not chemotaxis through FPR2. FPR1 and FPR2 are mainly expressed on the cell surface of phagocytic cells. Nevertheless, FPR3 is not expressed on human neutrophils, but it has been observed in eosinophils, monocytes, macrophages and dendritic cells, where it could be involved in the pathogenesis of allergic disease (Devosse et al., 2009). FPR3 is considered an orphan receptor since its ligands are not characterized yet. It was shown that it is insensitive to formylated peptides, but it is able to bind F2L, an endogenous 21- amino acid acetylated amino-terminal peptide (Migeotte et al., 2005). F2L binds FPR3 and induces monocyte intracellular calcium flux, ERK1/2 phosphorylation, and chemotaxis, and by increasing LPS-mediated IL-12 generation in dendritic cells, it inhibits their maturation (Kang et al., 2005) (Migeotte et al., 2005). Even if FPR2 and FPR3 share an identity of 83% of the amino acid (Bao, L., et al, 1992), they show some differences. In particular, phosphorylation is crucial for FPR2 desensitization, but the lack of phosphorylation did not result in increased or sustained responses, whereas FPR3, in resting cells, is characterized by a high degree of phosphorylation, which is only minimally enhanced by agonist activation. Another distinguishing feature of the two receptors was their subcellular localization in unstimulated cells. FPR2 is distributed equally on the plasma membrane, while FPR3 is localized in tiny intracellular vesicles and its intracellular distribution is related to a constitutive internalization which results unaffected by the C terminus phosphorylation (Rabiet et al., 2011). Since these receptors were firstly identified on the surface of the immune system cells and they recognize N-formylated peptides derived mainly from bacteria and pathogens, it was thought that they are only involved in the host defense against bacterial infection and in the clearance of damaged cells. However, it has been unraveled that these receptors were functionally expressed in other cell types and that both FPR2 and FPR3 were able to bind other kind of ligands supporting the idea that they are involved in other physiological and pathological function such as amyloidosis, Alzheimer's disease, prion disease, cancer and HIV (He & Ye, 2017).

2.7.1 Formyl peptide receptor 2

Formyl peptide receptor 2 (FPR2) is considered the most ubiquitous isoform of the FPRs family, resulting mainly expressed in cells of the bone marrow, immune system, gastro-intestinal tract, female organ tissues, endocrine glands, brain, liver, gallbladder, and pancreas (Krepel & Wang, 2019). FPR2 is both involved neutrophil chemotaxis and in the progression of several diseases (Snapkov et al., 2016; Xiang et al., 2016). Several FPR2 ligands can promote numerous cell functions such as chemotaxis, calcium flux, and phagocytosis (He & Ye, 2017). Moreover, depending on the nature of the ligands, FPR2 is able to modulate both pro-inflammatory processes and pro-resolving or anti-inflammatory pathways. In particular, bacterial and mitochondrial formylated peptides induce a pro-inflammatory response on FPR2, whereas annexin A1 (ANXA1) and lipoxin A4 (LXA4) are two anti-inflammatory FPR2 ligands (Stama et al., 2017). This duality in communicating opposing signals is also dependent on the receptor domains employed by pro-resolving or pro-inflammatory agonists (Dalli et al., 2013) , and the transition between FPR2-mediated pro-inflammatory and anti-inflammatory cell responses is due to receptor conformational changes following ligand binding (Cooray et al., 2013). To note, pro-resolving mediators such as ANXA1 and LXA4 reduce inflammation without impairing host defense against pathogens (Basil & Levy, 2016). Serum- amyloid alpha (SAA), instead, acts as a pro-inflammatory ligand on FPR2 by activating NF-kB pathway and increasing the production of pro-inflammatory proteins (Sack, 2018).

Although FPR2 recognizes formylated peptides derived from bacteria (PAMPs) or from damaged cells (DAMPs), it is also able to bind non-formylated peptides. Many of these non-formylated peptides derive from virus such as from the HIV-1 envelope (Braun et al., 2001) (Wood et al., 2014), hepatitis C virus, HKU-1 coronavirus, and herpes simplex virus (Lin et al., 2011; Mills, 2006) (Bellner et al., 2005). There are a few non-viral and non-formylated FPR2 microbe-derived agonists, such as *Enteroccocus faecium* strains (Bloes et al., 2012), as well as PAMP-derived agonists, such as those from *Helicobacter pylori* (Betten et al., 2001). Many diverse host-derived ligands have various biological consequences. Among these, amyloid-42 (Aβ-42), a well-documented peptide fragment in Alzheimer's illness, the prion peptide fragment PrP106-126, the neuropeptide pituitary adenylate cyclase-activating polypeptide 27, and the vasoactive intestinal peptide (Cattaneo et al., 2013a). Several host-derived FPR2 ligands were also identified such as the SHAAGtide sequence, various domain from the urokinase-type 1 plasminogen activator receptor, the F2L peptide derived from N-terminus of the heme-binding protein, and the chemokine-like peptide obtained from family with sequence similarity (Krepel & Wang, 2019). Interestingly, in addition to endogenous peptides, FPR2 binds also ligands secreted by damaged cells, synthetic agonists such as WKYMVm peptide (Kim et al., 2018) and peptides or lipids of different origin. FPR2 is mainly expressed on the surface of immune cells, but it localizes also onto cellular membrane of several nonphagocytic cells (Le, Y., et al, 2002) and onto nuclear membranes of CaLu-6 and AGS cells, where it is involved in gene expression

regulation (Cattaneo, F., et al, 2016). Once activated, FPR2 triggers the phosphorylation of several signaling proteins such as phospholipase C (PLC), phospholipase D (PLD), phosphatidylinositol-3-kinase (PI3K), protein kinase B (PKB or Akt), protein kinase C, p38MAPK, extracellular response kinase 1 and 2 (ERK1/2), and c-Src (Hodges et al., 2017) (Cao et al., 2023; Mottola et al., 2017) (L. Zhang et al., 2017), which regulate cell growth, proliferation, intracellular communication, apoptosis, survival, differentiation and other important intracellular functions (Ammendola et al., 2021). FPR2 stimulation results also involved in the phosphorylation of two non-signaling proteins named p47phox and p67phox which are crucially involved in NADPH oxidase activation and in turn in the ROS production (Iaccio, A., et al, 2009)(Cattaneo, F., et al, 2011).

2.7.1.1 FPR2 ligands

FPRs show the ability to bind a wide range of ligands, with different structure and chemical origin including both natural peptides both synthetic non-peptide compounds (Ye et al., 2009). FPRs are thus classified as a type of pattern recognition receptor (PRR) that recognizes pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) (Honda et al., 2017; Zhang et al., 2010). Among FPRs, FPR2 is considered the most versatile isoform of the family, since it can recognize different ligands of different origins. In fact, it binds both lipid and protein ligands. However, except for the eicosanoid LXA4 and the synthetic small-molecular weight ligands discovered via chemical library screen, the majority of FPR2 agonists are peptides. Many of these peptides are produced based on known protein sequences, but their physiological function in vivo must to be established. Several FPR2 agonists have been found and isolated from living organisms (Cattaneo et al., 2013a). FPR2 is also able to bind microbe-derive peptides, such as those derived from *Helicobacter pylori* (Betten et al., 2001) and HIV-1 (Cattaneo et al., 2013a) as well as mitochondrial peptides (Cao et al., 2023) . The ability of FPR2 to mediate a variety of biological effects may be due to distinct receptor domains recognize by different agonists.

2.7.1.2 FPR2 agonists

FPR2 ligands can be distinguished into: microbe-derived peptide, endogenous ligands and exogenous ligands. Among microbe-derived peptides, Hp(2-20) is a cecropin-like peptide derived from *Helicobacter pylori (H.pylori)*. By binding FPR2 and FPR3, this peptide is a chemoattractant in monocytes and it induces NADPH oxidase activation and so ROS production. It also induces chemotaxis, migration and proliferation of MKN-28 and AGS cell lines, which express FPR2 and FPR3 (de Paulis et al., 2009). FPR2 stimulation results in increased VEGF-A expression and secretion, p44/p42 MAPK activation, and in Akt and signal transducer and activator of transcription 3 (STAT3) phosphorylation (de Paulis et al., 2009). It also accelerates healing of rat

gastric mucosa after injury brought about by indomethacin at both the macroscopic and microscopic levels (de Paulis et al., 2009).

Other FPR2 ligands arise from HIV envelope proteins. These proteins include at least two sequences in glycoprotein gp120 and two in gp41. A synthetic peptide domain (F peptide), (EGSDTITLPCRIKQFINMWQE), localized in the V4-C4 region of gp120 of the HIV-1 Bru strain, is an agonist of FPR2. It induces chemotaxis and calcium mobilization in monocytes and neutrophils. In monocytes, F peptide reduces the expression of CCR5 and CXCR4 in a protein kinase C (PKC)-dependent manner, implying that activation of FPR2 by a peptide domain derived from HIV-1 gp120 may lead to cell desensitization to other chemoattractants (Le, Y., et al 2001). Another synthetic peptide (RIHIGPGRAFYTTKN) activates the FPR2 receptor in monocytes and neutrophils by being taken from the linear sequence of the V3 region of the HIV-1 envelope gp120 (MN strain) (Braun et al., 2001). It has been demonstrated that the binding of V3 on monocytes results in a decreased response to numerous chemokines that use multiple cell receptors, probably due to heterologous receptor desensitization. In particular, V3 stimulation induces the rapid phosphorylation of the chemokine receptor, CCR5, on serine residues through PKC activation. Furthermore, V3 peptide mobilizes Ca^{2+} and is an effective chemoattractant for human monocytes and neutrophils, but very slightly for T-lymphocytes (Braun et al., 2001). T21/DP107 peptide (NNLLRAIEAQHLLQLTVWGIKQLQARILAVERYLKDQ) is a leucine zipper-like domain localized in the amino terminus of the ectodomain of gp41. By binding FPR2 with high efficiency in human monocytes and neutrophils, it induces migration and calcium mobilization (Cattaneo et al., 2013a). However, it is unclear if soluble gp41 can interact with FPRs or if the binding to CD4, another HIV-1 fusion receptor, can favor its interaction with FPRs. It was thought that FPRs may have a role in the control of host innate immune responses found in AIDS patients, who have an initial activation of the immune system followed by gradual immunosuppression. N36 peptide (SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL) is located in the N-terminal heptad repeat region of HIV-1 gp41. By using FPR2 as a functional receptor, it induces migration and calcium mobilization in human monocytes. Moreover, F peptide and N36 peptide in human THP-1 monocytes, primary neutrophils and mouse leukocytes, increase the expression of endogenous tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which requires NFkB activation (Lin et al., 2007). Increased TRAIL expression in mice inhibited the development of transplanted mouse liver tumor cells considerably by triggering apoptotic cell death. These data underline the role of FPR2 and TRAIL in tumor immune surveillance and innate immunity and suggest a novel strategy for cancer therapy (Lin et al., 2007). FPR2, at nanomolar concentrations, is also activated by *S. aureus* toxins, which stimulate chemotaxis, calcium flux, and IL-8 production (Kretschmer et al., 2010).

As mentioned before, FPR2 binds N-formyl peptides that can derive not only by pathogens, but also by damaged mitochondria. Formyl-MMYALF is the mitochondrial human peptide that binds to the receptor with higher efficiency, causing migration of

FPR2-expressiong HL-60 cells as well as activation of NADPH oxidase producing ROS, that is an important in the host defense mechanism in phagocytes. An important category of FPR2 agonists includes three amyloidogenic polypeptides, serum amyloid A (SAA), the β-amyloid peptide 42 (Aβ42) and a peptide fragment of the aberrant human prion protein ($PrP_{106-126}$), linked to chronic inflammation and amyloidosis. SAA is a key acute-phase protein, normally present in the serum, even if its concentration can rise in response to a variety of traumas. In chronic inflammation, it can be enzymatically broken into pieces that precipitate to form amorphous amyloid fibril deposits, leading in amyloidosis (Sack, 2020).The 42-aminoacid form of Aβ42 is a self-aggregating peptide involved in the pathogenesis of Alzheimer's disease (AD). FPR2 plays an important role in AD, since by binding Aβ42, it induces a pro-inflammatory response in the brain of Alzheimer's patients (Iribarren et al., 2005; Le, Gong, et al., 2001). Moreover, FPR2 promotes internalization of this peptide to favor its uptake and fibrillary aggregation in mononuclear phagocytes (Iribarren et al., 2005). Brain tissue also releases protective substances that may counteract Aβ42's neurodestructive effect (Hashimoto, Niikura, Ito, et al., 2001; Hashimoto, Niikura, Tajima, et al., 2001). In particular, humanin (HN) is neuroprotective polypeptide able to bind FPR2, which protect neuronal cells from damage induced by Aβ42. In mononuclear phagocytes by using FPR2, it inhibits the aggregation and fibrillary formation and moreover, it protects cells from cytotoxicity and apoptosis caused by Aβ42, implying that HN competes with Aβ42 for binding to FPR2 (Ying et al., 2004). $PrP_{106-126}$ is a 21-amino acid fragment associated with prion disorders. In glial cells, it binds FPR2, inducing protein tyrosine phosphorylation, chemotaxis, calcium mobilization and increase the release of pro-inflammatory cytokines (IL-6, TNF-α) (Le, Yazawa, et al., 2001). This suggests that FPR2 is involved in the inflammatory pathology of prion disease.

LL-37 is a 37-residue mature antimicrobial peptide of the human cathelicidin hCAP18. LL-37 is present in neutrophil granules and epithelial cell surfaces. It is chemotactic for human neutrophils and T-lymphocytes that express FPR2, suggesting that LL-37 is involved in the innate and adaptive immunity by attracting neutrophils, monocytes, and T-cells to areas of microbial invasion through interaction with FPR2 (De et al., 2000). ANXA1 is a 37 kDa glucocorticoid-regulated phospholipid-binding protein with pro and anti-inflammatory activity that is partially mediated by FPR activation. In particular, its dual characteristics appear to be mediated by peptide derived from its N-terminus domain (Ac2-26 and Ac9-25), which are presumably produced in sites of inflammation (Tylek et al., 2021). ANXA1 is abundant in neutrophils, although it is also found in a number of other cell types (Ernst et al., 2004) . LXA4 is a novel arachidonic acid metabolite with anti-inflammatory and immunoregulatory properties, as demonstrated in many in vivo studies (Serhan et al., 2007). It works by binding FPR2 with high affinity, promoting arachidonate release as well as GTPase, cPLA2, PLD activity (Cattaneo et al., 2013a). LXA4 does not induce FPR2-dependent signaling, such as chemotaxis, ROS production and calcium mobilization (Forsman & Dahlgren, 2009) (Forsman et al., 2011).

Among all the synthetic peptide agonists for FPRs, a hexapeptide obtained from a random peptide library, Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm), was found to be a powerful activator for FPR1, FPR2 and FPR3 (Kang et al., 2005). It binds FPR2 with higher efficiency than the binding to FPR1 and FPR3 (Kang et al., 2005). As a result, it induces neutrophils and monocyte functions including chemotaxis, mobilization of complement receptor-3, cytokine release, and activation of NADPH oxidase, that result in the respiratory burst (Cattaneo et al., 2013a). MMK-1 (LESIFRSLLFRVM), another peptide discovered in a library screen, is a highly selective chemotactic agonist for FPR2 (Hu et al., 2001). CGEN-855A, a 21-amino acid anti-inflammatory peptide derived using a computational platform, was discovered to be an FPR2 and FPR3 agonist (He & Ye, 2017).

2.7.1.3 FPR2 antagonists

Since FPRs represent a potentially appealing pharmacological target, compounds that inhibit cell responses elicited by FPR agonists are expected to be useful in treating FPR-related illnesses. These molecules, which are typically derived from endogenous peptides and secondary metabolites, can directly interact with FPRs or interfere with their downstream signaling pathways. In particular, FPR2 has different antagonists. An example of FPR2 antagonist is an endogenous allergen uteroglobin (UG), known as Clara cell 10 kDa protein in humans. It is an anti-inflammatory and anti-chemotactic protein that is produced constitutively in mammalian airway epithelial cells. It inhibits fMLP-induced chemotaxis of monocytes and neutrophils (He & Ye, 2017). Urokinase receptor-derived cyclic peptide is a natural antagonist of FPR2. By screening hexapeptide libraries, it has been reported that several peptides compete with WKYMVm peptide for the binding to FPR2. Specifically, WRWWWW (WRW4) is identified as the most potent of these peptides with antagonist activity (Bae et al., 2004). A cell permeable peptide of ten amino acids coupled with a rhodamine group (RhoB-QRLFQVKGRR, **PBP10**) was recently reported to exhibit significant FPR2 inhibitory action. This peptide can completely inhibit ROS production mediated by FPR2, but not by FPR1 (Forsman et al., 2012). The pepducin F1Pal16, generated from a portion of FPR1's third intracellular loop, was discovered to block FPR2-mediated cellular response but not FPR1 signaling (Winther et al., 2015). Pam-(Lys-βNSpe)6-NH2 , a lapidated-peptide/-peptoid oligomer, was recently discovered to be a cross-species antagonist with FPR2 and mFPR2 selectivity (He & Ye, 2017). Pam-(Lys-βNSpe)6-NH2 and its structural analogs have been proposed as FPR2 allosteric modulators because they can inhibit neutrophil responses induced by the FPR2 pepducin F2Pal10, which is thought to act differently than conventional FPR2 agonists by anchoring to the receptor's intracellular loop. Quin-C7 has been reported as an FPR2-specific antagonist, however its activity at other inflammation-related receptors (such as C5aR and CXCR) has not been thoroughly investigated. However, further studies are required to analyze

off-target effects of these compounds and to determine specific action of these molecules on FPRs.

2.8 FPR2 signaling

FPR2 is stimulated by a wide range of ligands including proteins, peptides and lipids. Most of them, in addition to inducing chemotaxis, stimulate pro-inflammatory or pro-resolving pathways (He & Ye, 2017). Its ability to convey opposite signals depends on the nature of the ligands and on the diverse receptor's domain they used (Dalli et al., 2013) (Cooray et al., 2013). In particular, it has been observed that FPRs can form higher order structures which alter downstream intracellular signaling pathways by allowing effector domain colocalization, enhancing intracellular activation, or creating new ligand specificity. As regards FPR2, evidences demonstrated that it can form FPR1/FPR2 heterodimers as well as FPR2 homodimers that can influence its response to specific ligands (Tylek et al., 2021). The synthetic peptide WKYMVm, ANXA1 and LXA4 are anti-inflammatory ligands of FPR2 (Kim et al., 2019) (Perretti et al., 2002) (Galvão et al., 2020), whereas SAA and β-amyloid act as a pro-inflammatory agonists on FPR2 (Ye & Sun, 2015). FPR2 is also involved in cancer progression. Indeed, it is reported that the invasion of ovarian cancer cells necessitates FPR2 activation by the cathelicidin LL-37 (Coffelt et al., 2009), whereas gastric cancer cells require Hp(2-20) stimulation on FPR2 to promote cell migration and proliferation (de Paulis et al., 2009), ANXA1 is, instead, involved in the development and progression of astrocytoma. However, the FPR2 function in cancer progression and malignancy is still controversial and seems to be dependent on the nature of its ligands and on cell types, as reported by the observation that LXA4 reduces cell invasion in pancreatic cancer (Zong et al., 2016). FPR2 stimulation can induce the activation of several proteins kinases, and, in turn, the phosphorylation of many cytosolic signaling proteins (Ammendola et al., 2021) (Cattaneo et al., 2019) involved in the regulation of several cellular function such as proliferation, differentiation, migration, communication, and other fundamental functions. In particular, it induces the activation of protein kinase C (PKC) isoforms, phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), mitogen-activated kinase (MAPK), p38MAPK, PTEN, PTP-PEST and DUSP3/VHR (Cattaneo et al., 2013a) (Cattaneo et al., 2010) (Cattaneo et al., 2014; Leoni et al., 2013; Wentworth et al., 2011). Moreover, some of the FPR2-dependent phosphorylated molecules are non-signaling proteins, such as the NADPH oxidase complex cytosolic components p47phox and p67phox, whose phosphorylation needs FPR2-dependent activation of ERKs, PKCα and PKCδ (Cattaneo et al., 2011). Once activated NADPH oxidase complex produces ROS. At low concentration, ROS can act as second messengers by transactivating tyrosine kinase receptor (TKRs) (Cattaneo et al., 2014). In previous studies it has been demonstrated that, in CaLu-6 cells, FPR2 stimulation with W peptide causes the EGFR transphosphorylation in a NADPH oxidase-ROS-Src dependent manner CaLu-6 cell line. EGFR phosphotyrosine residues, once activated, serve as docking sites for the

recruitment, phosphorylation, and activation of the transcriptional factor Signal Transducer and Activator of Transcription 3 (STAT3) (Cattaneo et al., 2011). FPR2 activation causes phosphorylation of Hepatocyte Growth Factor Receptor (c-Met) residues Y1313,Y1349, and Y1356 in the human prostate epithelial cell line PNT1A, activating STAT3, PLC-1/PKC, and PI3K/Akt pathways (Cattaneo et al., 2013b). Characterization of cell phosphorylation status is critical in the study of intracellular signaling cascades, the comprehension of the molecular mechanisms behind human illnesses and, ultimately, the proper pharmacological strategy (Marino et al., 2019). Indeed, these signaling cascades regulate various cellular activities, and their dysfunction contributes considerably to the development of human illnesses.

Recently, to further investigate intracellular signaling cascades activated in FPR2-stimulated CaLu-6 cells we performed a phosphoproteomic analysis and we identified 290 phosphoproteins and 53 unique phosphopeptides mapping on 40 proteins. In this study, we demonstrated that WKYMVm peptide stimulation in CaLu-6 cells induces the selective phosphorylation on Ser82 of Heat Shock Protein-27 (HSP-27), Ser139 of Mini-Chromosome Maintenance protein 2 (MCM2), Ser608 of Retinoblastoma (Rb), Ser339 of Oxidative-Stress-Responsive kinase 1(OSR1) and Ser170 of Myristolated Alanine-Rich C-Kinase Substrate (MARCKS) (Cattaneo et al., 2019). Similarly, also ANXA1 stimulation increases phosphorylation of HSP-27, OSR1 and MARCKS. Moreover, both agonists trigger the phosphorylation of p38MAPK, PI3K and PKCδ, the kinases upstream to HSP-27, OSR1 and MARCKS respectively, in a NOX2-dependent manner (Ammendola et al., 2021). Therefore, the versatility and heterogeneity of signaling cascades triggered by FPR2 means that it can be considered a novel promising therapeutic target for the treatment of diseases associated with inflammation.

2.9 Therapeutic approach on FPR2

FPR2 is considered an interesting and promising therapeutic target since it is functionally expressed in several cell types, including endothelial and epithelial cells, fibroblasts and neuronal cells, besides of immune cells and is also able to bind a wide range of ligands. Furthermore, FPR2 is crucially involved in many diseases where it plays different roles. In cells, FPR2 can be involved in pro-inflammatory response or in pro-resolving response, depending on the ligands and on particular receptor domain recognized by them. It has been observed that ANXA1 binds and activates FPR2 during influenza and it is responsible of virus adsorption and replication in host cells (Tcherniuk et al., 2016). Therefore, FPR2 has been considered a potential target to treat influenza. In fact, it has been reported that by blocking FPR2 with specific antagonists such as WRW4, mice are protected against infections, due to inhibition of viral replication and deleterious inflammation of the lungs. Furthermore, FPR2 antagonists are expected to provide long- term protection because they reduce inflammation, which is responsible for tissue harm later in the infection (Alessi et al., 2017).

A therapeutic role for FPR2 has been evaluated in asthma. Asthma is a progressive condition, and it has been found that the severity of the disease is linked to a decrease in the production of pro-resolving mediators in these patients. Since FPR2 is also able to induce a pro-resolving response, it has been hypothesized that it can be involved in the chronic, non-resolving inflammatory conditions of the airways in asthma, in particular the development of selective agonists seems to be a therapeutic approach to treat patients with moderate to severe asthma (Visaga et al., 2021).

Atherosclerosis is a chronic inflammatory condition that primarily affects the arteries' intimal layer, particularly at blood artery bifurcation points. Several evidence supports the idea that FPR2 plays a pathogenic role in atherosclerosis. For instance: (i) it is expressed in macrophages in human atherosclerotic lesions, where it speeds up the development of atherosclerosis via effects on bone marrow-derived cells (Petri et al., 2015); (ii) FPR2 expression in the carotid artery is linked to clinical indications of cerebral ischemia and a more persistent plaque phenotype in atherosclerosis (Petri et al., 2015); (iii) FPR2 expression in smooth muscle cells (SMC) is associated with increased collagen formation and maturation, as well as decreased collagen degradation pathways (Petri et al., 2015). Therefore, it has been investigated the potential therapeutic role of FPR2 to treat this condition.

In vivo administration of the pro-resolving ANXA1 mimicking peptide Ac2-26 significantly reduces atherosclerotic lesion sizes and macrophages accumulation in an FPR2 dependent manner, and delivery of nanoparticles containing the pro-resolving ANXA1 mimicking peptide Ac2-26 reduces experimental atherosclerosis in the presence of a functional FPR2. In addition, other pro-resolving ligands of FPR2 exert a protective role in atherosclerosis. WKYMVm peptide, a synthetic anti-inflammatory FPR2 agonist, by recruiting endothelial progenitor cells, contributes to neovascularization (Choi et al., 2015; Heo et al., 2014), promotes re-endothelializations, and blocks restenosis (Jang et al., 2015), demonstrating therapeutic activity (Choi et al., 2016). Interestingly, FPR2 is also involved in myocardial infarct. For example, ANXA1 and N-terminal peptides exert a protective role in several model of ischemia riperfusion injury (IRI) (Ansari et al., 2018). In fact, recombinant ANXA1 infusion reduces myocardial infarct size (D'Amico et al., 2000), while Ac2-26 therapy reduces infarct size, myeloperoxidase (MPO), and IL-1β levels (La et al., 2001). In rats, intracerebroventricular infusion of Ac2-26 reduces stroke volume and cerebral edema (Relton et al., 1991) by inhibiting leukocyte-endothelial interactions (Smith et al., 2015). In IRI, FPR2 promotes inflammatory resolution by interacting with the pro-resolving lipid mediators RvD1. In fact, it has been observed that RvD1 administration, prior to ischemia-reperfusion, prevents inflammatory cascades by inhibiting the release of TNF-α, IL-6 and MPO levels and it also reduces apoptosis through Akt activation (Zhang et al., 2015) and attenuates IR-induced damage. These effects depend on FPR2 activation, as demonstrating by silencing FPR2 expression or by FPR2 antagonists (Kang & Lee, 2016). Since FPR2 is could represent a possible target for IRI, a variety of new ligands with protective properties have been discovered. These include the FPR2

agonist CGEN-855A, which has been shown to have cardioprotective effects in rat and mouse myocardial IRI, similar to the ANXA1 mimetic peptide Ac2-26 (Gavins et al., 2003). Furthermore, 15-epi-16-(p-fluorophenoxy)-LXA4-methyl ester [15-epi-16-(FPhO)-LXA4-Me] is an FPR2 agonist that protects against renal IRI via regulating cytokine and chemokine expression as well as neutrophil recruitment. Cmpd17b is an FPR1/FPR2 agonist with a bias that lowers the inflammatory responses associated with reperfusion after an acute MI (Qin et al., 2017). Finally, via binding FPR2, the new 3-oxa-aspirin-triggered 15-epi-lipoxin analogues ZK-994 and ZK-142 suppress neutrophil accumulation in murine hind-limb IRI-induced second organ lung injury (Bannenberg et al., 2004).

FPR2 plays also an important role in survival, structure, and function of cardiometabolic and renal syndrome. Considering that FPR2 is involved in cardiovascular diseases, as well as in inflammation diseases, several FPR2 agonists have also been tested in humans (Corminboeuf & Leroy, 2015; Perretti et al., 2015). In fact, synthetic lipoxin mimetics have been studied in preclinical settings, and a phase I study with ACT-389949, a small-molecule FPR2 agonist, has been done. FPR2 agonists are particularly useful in treating heart failure rather than traditional inflammatory disorders. Indeed, a Phase I study for the drug BMS-986235, a selective FPR2 agonist with tissue-protective characteristics in experimental heart failure, has been completed (Asahina et al., 2020). ANXA1 and its peptides are used for treatment of many diseases associated with acute or chronic inflammation. Interestingly, ANXA1 protects peripheral organs from hyperglycemia and hyperlipidemia-induced damage and dysfunction. Nonetheless, human translational studies have yet to be done. In preclinical settings, however, ANXA1 has a direct effect on cardiac macrophage polarization in the post-infarct heart by inducing a proangiogenic macrophage phenotype (Ferraro et al., 2019).

These findings indicate that pro-resolving mediators have positive effects via FPR2, which has implications for a variety of cardiovascular disorders and inflammation-related pathologies. For example, prolonged inflammatory reactions in atherosclerosis may be caused by a failure in inflammation resolution mediated by reduced synthesis of pro-resolving lipid FPR2 ligands as well as the unmasking of proinflammatory FPR2 signaling. Restoring pro-resolving FPR2 signaling may provide unique therapeutic options for both preventing atherosclerosis progression and enhancing the stability of atherosclerotic plaque. Therefore, pro-resolving effects triggered by FPR2 anti-inflammatory ligands can be considered an effective potential therapeutic approach to combine with current therapies.

3. Aims of the work

FPR2 is the most ubiquitous and promiscuous member of the Formyl Peptide Receptor family and is involved in many physiological and pathological conditions. To note, FPR2 exerts a dual role during inflammation, by modulating proinflammatory or proresolving response, depending on the nature of its agonists and on the different receptor domain recognized by specific ligand. As a member of the GPCR superfamily it is expressed onto the membrane of cells, but, interestingly it is also found in the nuclear membrane of AGS and CaLu-6 cells, where it is involved in the regulation of gene expression (Cattaneo et al., 2016). Once stimulated, FPR2 induces the phosphorylation and activation of several protein kinases and phosphatases, including protein kinase C isoforms (PKC), phosphoinositide-3-kinase (PI3K), protein kinase B (Akt), mitogen-activated protein kinase (MAPK), p38MAPK, PTEN, PTP-PEST, and DUSP3/VHR (Cattaneo et al., 2013a; Weiß & Kretschmer, 2018) (Cattaneo et al., 2010) (Cattaneo et al., 2014) (Leoni et al., 2013) (Wentworth et al., 2011), as well as the phosphorylation of two non-signaling proteins p47phox and p67phox which are required for NADPH oxidase-dependent reactive oxygen species (ROS) generation (Ammendola et al., 2004; Iaccio et al., 2009). Due to ROS production, FPR2 can transactivate Tyrosine Kinase Receptors (RTKs) (Cattaneo et al., 2011; Cattaneo et al., 2013b).

Recently, a phospho-proteomic analysis in FPR2- stimulated CaLu-6 cells unraveled 290 differentially phosphorylated protein in FPR2 stimulated cells, and, the largest part of these phosphoproteins are involved in the regulation of the energetic metabolism. This new emerging role exerted by FPR2 in cell metabolism has never been characterized. Therefore, the aims of this study are:

- To identify metabolic pathway activated in CaLu-6 cells stimulated with FPR2 agonists;
- To evaluate the role of FPR2 in metabolic reprogramming.

We apply a metabolomic approach to analyze metabolic pathways activated in human lung anaplastic cancer CaLu-6 cells stimulated with WKYMVm peptide or ANXA1, two FPR2 agonists.

4. Materials and Methods

4.1 Cell culture and reagents

CaLu-6 and p22phox Crispr/Cas9 CaLu-6 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp., Carlsband, CA, USA) at 37° C and 5% CO_2. Cells were grown to 70% confluence. Then they are serum starved for 24 hours and treated or not with 10µM WKYMVm peptide (Primm, Milan, Italy) or with 10nM ANXA1for different times, as reported in the figures. CaLu-6 cells were pretreated with 10µM WRWWWW (WRW4) (Primm, Milan, Italy) for 15 minutes or with 100µM apocynin (Sigma Chemical, St Louis, MO, USA) for 2 hours, or with 10µM PP2 and PP3 (Calbiochem, La Jolla, CA,USA) for 45 minutes, or with 3µM GSK1904529A (MedChemExpress, Monmouth Junction, NJ, USA) for 2 hours, or with 5µM LY2874455 (MedChemExpress, Monmouth Junction, NJ, USA) for 2 hours, or with 2µM AG1478 (Calbiochem, La Jolla, CA, USA) for 60 minutes, or with 0.5µM wortmannin (Calbiochem, La Jolla, CA, USA) for 60 minutes, or with 10µM LY294002 (Calbiochem, La Jolla, CA, USA) for 60 minutes, or with 50µM PD09805 for 90 minutes, or with 10µM SB203580 for 1 hour, before WKYMVm or ANXA1 stimulation.

4.2 p22phox Crispr/Cas9 double-nickase CaLu-6 cells

p22phox $^{Crispr/Cas9}$ cells were obtained by transfecting CaLu-6 cells with Double Plasmid control or with Double Nickase plasmid (Santa Cruz Biotechnology, Irvine, CA, USA), following the manufacturer's instructions. In a single of a multi-well tissue culture plate, $1.5x10^5$ CaLu-6 wild type cells were seeded in DMEM containing 10%FBS without antibiotics for 24 hours. The cells were then transfected with 3µg of p22phox Double Nickase Plasmid DNA or with 3µg of control Double Nickase plasmid as a negative control using 10µL of UltraCruz® transfection Reagent, as indicated by the supplier. Double Nickase Plasmid is a pool of plasmid pairs, each coding for a mutant Cas9 nuclease D10A and a 20-nt target-specific guide RNA. One of the plasmids in the pair has a puromycin resistence gene for selection, while the other contains a green fluorescence protein (GFP) marker to visually confirm transfection. For 48 hours, the culture medium was changed to a selective medium containing 1µg/mL puromycin (Santa Cruz, Biotechnology, Dallas, TX, USA). Transfected cells were isolated by culturing cells in puromycin media for 5 days and the medium was changed every 2 days. To evaluate p22phox expression, single cell clones were tested by Western blot analysis. Colonies knockout for the p22phox gene were isolated to obtain p22phox$^{Crispr/Cas9}$ pool cells (p22phox$^{Crispr/Cas9}$), whereas control Double Nickase

plasmid-transfected cells, which express p22phox, were recovered to obtain CaLu-6 control $^{Crispr/Cas9}$ cells.

4.3 Metabolomic Analysis by liquid chromatography-mass spectrometry

LC-MS was done on growing and serum-starved CaLu-6 cells stimulated or not with WKYMVm in the presence or absence of WRW4. $2x10^4$ cells were seeded in a 48-well plate and serum-starved for 24 hours before the treatments the next day. After treatments, cells were lysed in 400µl of a 1:1 prechilled MetOH:H_2O solution. The samples were vortexed, stored on ice for 20 minutes, and centrifuged again at 10,000g for 10 minutes at 4°C. Supernatants were collected and then dried in a SpeedVac concentrator system (Thermo Scientific) set at room temperature. These supernatants were reconstituted with 125µl of methanol/acetonitrile/water (50:25:25). Extracted metabolites were analyzed in positive and negative modes under the following conditions using an ACQUITY UPLC system online linked to a Synapt G2-Si QTOPF-MS (Waters Corporation, Milford, MA, USA): reverse phase ACQUITY UPLC CSH C18 (1.7m, 100 2.1 mm^2) column (Waters), 0.3mL/min flow rate, mobile phases of acetonitrile/H_2O (60:40) containing 0.1% formic acid and 10mM ammonium formate (Phase A), and isopropanol/acetonitrile (90:10) containing 0.1% formic acid and 10mM ammonium formate (Phase B). Peak detenction, metabolite identification, and quantification were carried out as previously described (Sommella et al., 2019), using internal standard and calibration curves to match experimental data. As previously described (Riccio et al., 2018) (Badolati et al., 2018), data analysis and heatmaps were generated using the online software MetaboAnalyst (https://www.metaboanalyst.ca).

4.4 2-NBDG glucose uptake assay on CaLu-6 cells

CaLu-6 cells ($5x10^3$ per well) were plated in a black, clear bottom, 96-well microtiter plate (Perkin Elmer, Waltham, USA) in a final volume of 100µl of growth media. After 24 hours, the culture medium was carefully removed and replaced with 100µl of HBSS containing 100µM 2-deoxyglucose (2-DG), 0.4 g I^{-1} BSA , and 1.3mM $CaCl_2$ (in the absence of any growth factors or FBS) and incubated with WKYMVm at the indicated concentrations for the indicated times in the presence or absence of WRW4. Plates were then incubated for 1 hour at 37°C. The treatments were carried out in triplicate, and the results are the media of three independent experiments. The medium was replaced with HBSS containing 100µM 2-DG and 6µM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG). Plates were incubated for 45 minutes with the fluorescent probe before being rinsed twice in PBS. The uptake of 2-NDBG was measured using a Perkin Elmer Envison 2015 multiplate reader (Perkin Elmer) and the following parameters: excitation 471 nm, emission 529 nm, and monochromator cut off 360 nm. Cells were fixed in 3.7%

paraformaldehyde for 30 minutes before being permeabilized in 0.1% Triton X100 in PBS and stained with the nuclear dye DAPI (30µM). This second fluorescence measurement was used for normalization, since it correlates with the total number of cells in each well. DAPI fluorescence was taken up by using the following parameters: λ excitation 351 nm and λ emission 450 nm. Data reported of glucose uptake are the ratio between intracellular 2-NBDG fluorescence and DAPI fluorescence ± s.d.

4.5 Protein extraction and Western Blot

Protein lysates were purified from 24 hours serum- starved CaLu-6 or p22phox$^{Crispr/Cas9}$ CaLu-6 cells stimulated or not with 10µM WKYMVm or ANXA1, in presence or absence of the above mentioned selective inhibitors. Whole lysates were obtained by scraping cells with ice cold RIPA buffer which contains: 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 0.25% sodium deoxycholate, 1mM NaF, 10µM Na_3VO_4, 1mM phenyl-methyl-sulfonyl-fluoride, 10µg ml^{-1} aprotinin, 10µg ml^{-1} pepstatin, 10µg ml^{-1} leuptin, as described before (Schiattarella et al., 2018). Membrane lysates were isolated as described above (Cattaneo et al., 2016). Lysates were prepared using a hypotonic buffer comprising 10mM Tris-HCl, 1mM CaCl2, 150mM NaCl, 1 mM phenyl-methyl-sulfonyl-fluoride, and a protease inhibitor cocktail (10µg ml^{-1} aprotinin, 10µg ml^{-1} pepstatin, 10µg ml^{-1} leuptin) (Buffer II) and then they were centrifuged at 400 x g for 10 minutes at 4°C, in order to separate membrane and cytosolic fraction. Membrane fraction extracted was incubated overnight at 4°C in constant agitation with a buffer that contains 125mM Tris-HCl, 1mM phenyl-methyl-sulfonul-fluoride, 1% Triton X100, and the protease inhibitor cocktail (Buffer II). Protein concentrations were determined using the Bio-Rad protein assay (BioRAD, Hercules, CA, USA). Western blot analysis was done on whole or membrane lysates as previously disclosed (Pavone et al., 2011). The ECL chemioluminescence reagent kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was used to detect antigen-antibody complexes and protein were quantified using densitometry (Chemidoc, Bio-Rad).

Anti-GAPDH (SC-47724), anti-Tubulin (SC-8035), anti-Na K ATPase (SC-48345), anti-GLUT4 (SC-53566) and anti-phospho-c-Myc (S62) (SC-8000-R) were purchased from Santa Cruz Biotechnology (Irvine, CA, USA). Anti-phospho- IGF-IR (Y1131/1146), anti-phospho-PFKBP2 (S483), anti-phospho-FRS2 (Y436), anti-phospho-PDH (S293), anti-phospho-LDH (Y10), anti-phospho-c-Src (Y416), anti-ASCT2, anti-phospho-CAD (S1859), anti-phospho-cPLA2 (S505), anti-phospho-5-LOX(S271), anti-phospho-5-LOX(S663) were from Cell Signaling Technology. Anti-HIF1α (NB100-105) was bought from Novus Biologicals (Centennial, CO, USA). Goat-anti-mouse (bs-0296G-HRP) and goat-anti-rabbit (bs-0295G-HRP)

were purchased from Bioss Antibodies (Woburn, MA, USA). Each experiments and the relative densitometric quantification was performed at least three times.

4.6 Lactate assay

Lactate concentration was determined in CaLu-6 cell culture medium using the Lactate-Glo Assay (Promega) according to the manufacturer's instructions. In particular, $1.5x10^4$ cells were plated in a 96-well plate. After a day, cells were serum starved for 24 hours, and then preincubated or not with 10μM WRW4 for 15 minutes and stimulated or not with 10μM WKYMVm for 24 hours. For each experimental point, five microliters of medium were withdrawn and diluited in 95μl of PBS. 50μl of diluited media was transferred to a 96-well assay plate for each experimental point, and 50μl of lactate detection reagent was added. Before recording luminescence, the assay plate was shaken for 1 minute to mix the reagents and incubated for 60 minutes at room temperature. DMEM represented the negative control of the experiment. A Synergy H1 microplate reader (BioteK,VT, USA) was used to measure luminescence. The results are the mean of three independent studies, with each experimental point examined in triplicate.

4.7 Seahorse XF analysis

The Seahorse XF Glycolytic Rate Assay Kit (Agilent, CA, USA) was used to determine the extracellular acidification rate (ECAR). CaLu-6 cells were cultured in the XF-24 cell culture plates at 20 000 cells per well and after attached overnight, they were serum starved for 24 hours. Cells were treated with 10μM WKYMVm for 24 hours followed by Seahorse Assay. The medium was then changed to Seahorse XF DMEM medium ph 7.4 (Agilent), supplemented with 25mM glucose, 4mM L-glutamine, and 2mM pyruvate, and incubated for 1 hour in a CO_2-free incubator at 37°C. An XF-24 Analyzer (Agilent) was used to measure ECAR in real time.

4.8 Mitochondrial Membrane Potential Assay

The mitochondrial membrane potential ($\Delta\psi m$) was measured in CaLu-6 cells using Mitotracker ® Red CMXRos (Thermo Fisher Scientific) dye, as directed by the manufacturer. Before applying the dye, CaLu-6 cells were washed twice in PBS. Cells were treated with the probe for 45 minutes at 37°C and 5% CO_2. Followed that, the cells were washed three times in DMEM and once in PBS before being fixed in 3.7% formaldehyde for 30 minutes, permealized in 0.1% Triton X100 in PBS, and stained with the nuclear dye DAPI. Mitochondrial fluorescence was evaluated using a Perkin Elmer Envision 2015 Multiplate reader (Perkin Elmer) and

the following parameters: λ excitation 579nm, λ emission 599 for MitoTracker, λ excitation 351nm, and λ emission 450nm for DAPI. For normalization, the total number of cells in each well was used. The results represent the means of three independent experiments with each experiment analyzing each experimental point in triplicate.

4.9 $NADP^+$/NADPH Assay

To calculate $NADP^+$, NADPH and their ratio, $NADP^+$/NADPH assay was performed according to the manufacturer's instructions (Elabscience, Houston, TX, USA). CaLu-6 cells were serum-starved for 24 hours before being stimulated or not with 10µM WKYMVm or 10nM ANXA1 for various times, in other experiments, serum-starved cells were pretreated with WRW4 before being activated with WKYMVm or ANXA1. The OD value at 450nm was used to quantify $NADP^+$ and NADPH in a colorimetric assay. The results represent the means of three independent experiments, with each experiment analyzing each experimental point in triplicate.

4.10 Transketolase Activity Assay

The enzymatic activity of transketolase (TKT) was evaluated in CaLu-6 cells using a Transketolase activity assay kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. In brief, $4x10^5$ CaLu-6 cells were serum-starved for 24 hours before being treated with 10µM WKYMVm or 10nM ANXA1 for varying periods of time. In other experiments, serum-depleted CaLu-6 cells were pretreated with WRW4 before being activated with WKYMVm or ANXA1. TKT activity was evaluated by measuring the fluorescence (RFU) released from the conversion of a non-fluorescent probe to a fluorescent probe (λEx= 535/ λEm=587) in each well of a 96-well plate for each sample. All samples and standards were tested twice. The results are the average of three distinct experiments.

4.11 Statistical analysis

The unpaired t-test or one-way analysis of variance (ANOVA) were used to compare the means of two-independent groups of experiments. GraphPad Prism 7(GraphPad Software Inc., San Diego, CA, USA) was used in order to compare more than two experiments. All data presented are the results of at least three independent experiments and are shown as means, standard error mean (SEM). A *p* value of less than 0.05 was considered to be statistically significant.

5. Results

5.1 FPR2 stimulation contributes to glucose metabolism

Our metabolomic analysis of CaLu-6 cells stimulated or not with WKYMVm peptide showed that treated cells had higher levels of metabolites involved in glucose metabolism, such as glucose-6-phosphate, fructose 1,6-bis-phosphate (F1,6BP), glyceraldheide 3-phosphate (GA3P) and lactate. Moreover, the preincubation with FPR2 antagonist, WRW4, significantly reduced this increase, suggesting that FPR2 stimulation promotes glucose oxidation via glycolysis (**Figure 7A**). In glycolysis, glucose is converted into pyruvate, which in aerobic conditions, enters into the mitochondria, where it is converted into Acetyl-coenzyme A (CoA) by pyruvate dehydrogenase complex (PDC). This can fuel the tricarboxylic acid (TCA) cycle, which in turn feeds the mitochondrial electron transport chain to produce energy. However, pyruvate can also be converted into lactate by lactate dehydrogenase (LDH). Interestingly, our metabolomic analysis showed a significant increase of lactate in FPR2-stimulated cells (**Figure 7A**). In malignant cells, this reaction characterizes the aerobic glucose utilization typical of the Warburg effect in cancer cells (Koppenol et al., 2011). Therefore, we analyzed glucose uptake in FPR2-stimulated CaLu-6 cells and we observed that stimulation with 10μM WKYMVm enhanced glucose consumption in both a time-(**Figure 7B**) and FPR2- (**Figure 7C**) dependent manner. The increased glucose utilization is considered a known hallmark of cancer cells. Glucose uptake is mediated by members of the transmembrane glucose transporter family, which includes facilitative glucose transporters (GLUTs), sodium-glucose co-transporters (SGLTs), and SWEET family transporters, which are abundant in plants (Martinez & Scafoglio, 2020). The GLUT family comprises 14 members, which are divided into three classes depending on their structure. GLUT1 expression is enhanced in cancer cells by Src, Ras, Myc and Akt (Osthus et al., 2000; Sasaki et al., 2012) (Barron et al., 2016; Barthel et al., 1999), and it is repressed by p53 (Schwartzenberg-Bar-Yoseph et al., 2004). GLUT4 is regulated by insulin (Suzuki & Kono, 1980; Wardzala & Jeanrenaud, 1981) and it is expressed in many cancer cells (Mao et al., 2019; McBrayer et al., 2012; Shibata et al., 2005). We evaluated GLUT1 and GLUT4 membrane translocation in WKYMVm stimulated cells and we observed an increased expression of GLUT4 in a time-dependent manner (**Figure 7D)** that is significantly reduced by WRW4 pretreatment (**Figure 7E**). We did not observe the same effect on GLUT1 translocation. In several studies it is reported that GLUT4 trafficking to the plasma membrane is regulated by phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Hou et al., 2019).

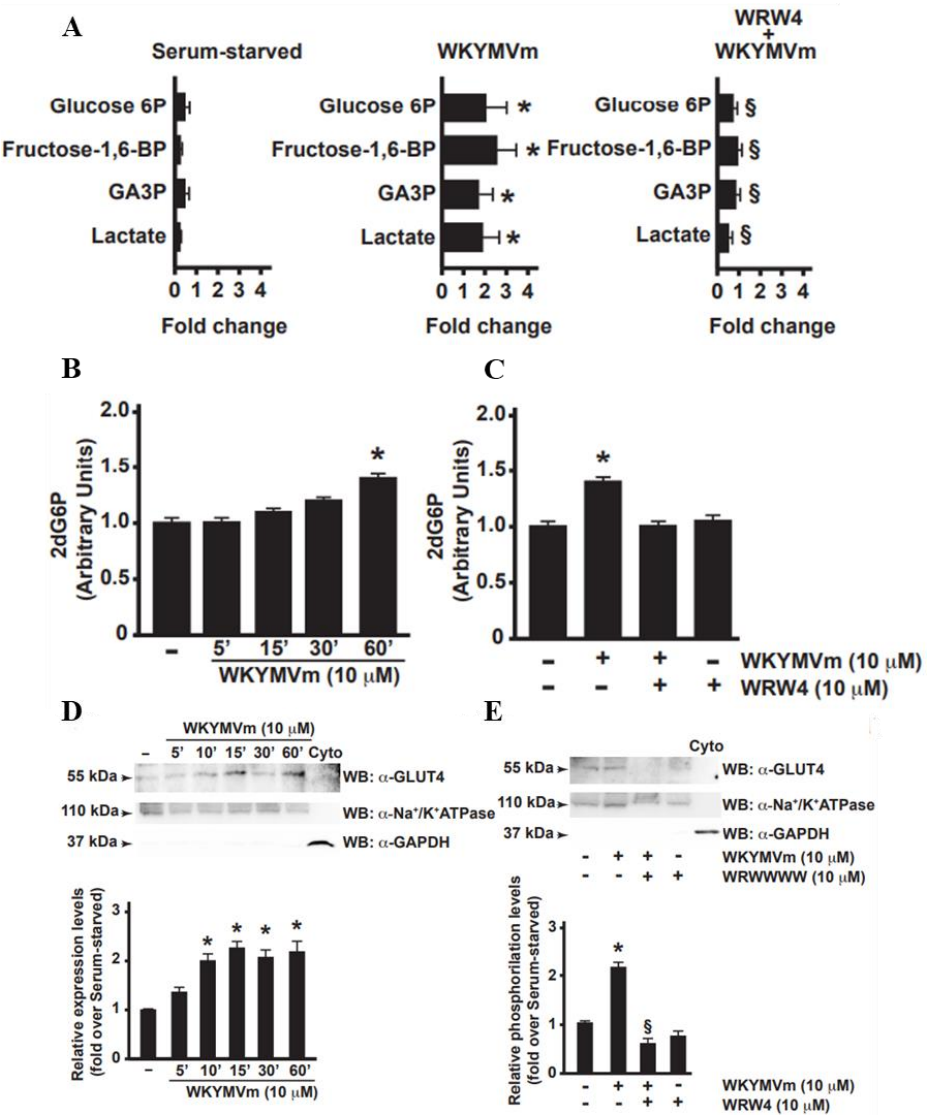

Figure 7. FPR2 improves glucose metabolism and GLUT4 membrane localization. FPR2 stimulation increases metabolites involved in glucose metabolism (A). CaLu-6 cells were serum starved for 24h and then stimulated or not with 10μM WKYMVm for 1 h in the presence or absence of WRWWWW (WRW4). FPR2 stimulation triggers glucose uptake (B,C). CaLu-6 cells were grown to 80% confluence before being exposed to 100μM for the indicated times, in the presence or absence of WRW4. 2-NBDG uptake was measured in a Perkin Elmer Envision 2105 multiplate reader. The results are the average of three independent experiments, with each point examined in triplicate. *$p < 0.05$ compared to unstimulated cells. GLUT4 translocation is regulated in a time (D) and FPR2 (E) dependent manner.CaLu-6 cells were serum depleted for 24h before being stimulated for 5, 10, 15, 30 and 60 min with WKYMVm (D), or preatreated with WRW4 (E). Sixty micrograms of membrane lysates were immunoblotted with anti-GLUT4 antibody (α-GLUT4) . Anti-Na^+/K^+ATPase antibody (α-Na^+/K^+ ATPase) was employed as a control for protein loading. Anti-GAPDH (α-GAPDH) was used as a cytosolic protein control. Data represents five different experiments. *$p<0.05$ compared to unstimulated cells. Glucose 6P: glucose-6-phosphate; fructose 1,6-BP: fructose 1,6.bis-phosphate; GA3P: glyceraldheide 3-phosphate.

5.2 FPR2 signaling transactives IGF-IRβ/ IRβ in a NOX2-dependent manner

FPR2 is able to induce the activation of several protein kinases and phosphatases, RTKs and PTPs (Cattaneo et al., 2014) (Cattaneo et al., 2011; Cattaneo et al., 2013b) (Ammendola et al., 2021; Cattaneo et al., 2010; Cattaneo et al., 2013a; Cattaneo et al., 2019). As a result of FPR2-mediated RTKs transactivation, cytosolic phosphotyrosine residues of RTKs serve as docking sites for recruitment and activation of the STAT3, PLC-1/PKC, and PI3K/ Akt pathways in various cell types (Cattaneo et al., 2013b) (Cattaneo et al., 2011). Because GLUT4 is the insulin-regulated member of the glucose transporter family, we investigated the ability of FPR2 to transactivate insulin-like growth factor-I receptor (IGF-IRβ) and/or insulin receptor (IRβ). IGF-IRβ is characterized by three main autophosphorylation sites Y1131, Y1135 and Y1136 (Hernández-Sánchez et al., 1995) that are necessary for kinase activation. IRβ is functionally and structurally identical to IGF-IRβ, including the presence of an analogous tyrosine cluster (Y1146, Y1150, Y1151). By using a monoclonal phospho-antibody against IGF-IRβ/ IRβ we observed a time dependent increase of IGF-IRβ phosphorylation (**Figure 8A**). By pretreating cells with WRW4 before stimulation, we found a decrease in IGF-IRβ phosphorylation (**Figure 8B**), suggesting that it depends on FPR2 activation.

It has been already demonstrated that FPR2 stimulation induces NOX2 activation (Ammendola et al., 2021; Cattaneo et al., 2011) (Cattaneo et al., 2019) and that FPR2 transactivates RTKs in a NOX2-dependent manner (Cattaneo et al., 2013b). In order to evaluate the involvement of NOX2 in IGF-IRβ transactivation, we pretreated cells with apocynin, which inhibits p47phox translocation and its interaction with p22phox, before WKYMVm stimulation and we observed a significant reduction in IGF-IRβ phosphorylation (**Figure 8C**). We created a CaLu-6 cell line that expresses a non-functional form of p22phox (p22phox$^{Crispr/Cas9}$) by using CRISPR/Cas9-based genome editing (Ammendola et al., 2021). Notably, WKYMVm stimulation of these cells failed to trigger IGF-IRβ/IRβ phosphorylation (**Figure 8D**), indicating that ROS are signaling intermediates in RTK transactivation (Catarzi et al., 2005; Fischer et al., 2004) (Bae et al., 1997; Sundaresan et al., 1995). Since FPR2-mediated IGF-IRβ transactivation requires NOX2 activation, we evaluated the role of NOX2 in GLUT4 translocation. Therefore, we preincubated cells with the NOX-specific inhibitor apocynin, before WKYMVm stimulation, (**Figure 8E**) and we stimulated p22phox$^{Crispr/Cas9}$ cells with the FPR2 agonist (**Figure 8F**). In both cases, we observed a significant reduction in GLUT4 translocation, suggesting that GLUT4 and IGF-IRβ require NOX2-dependent ROS production.

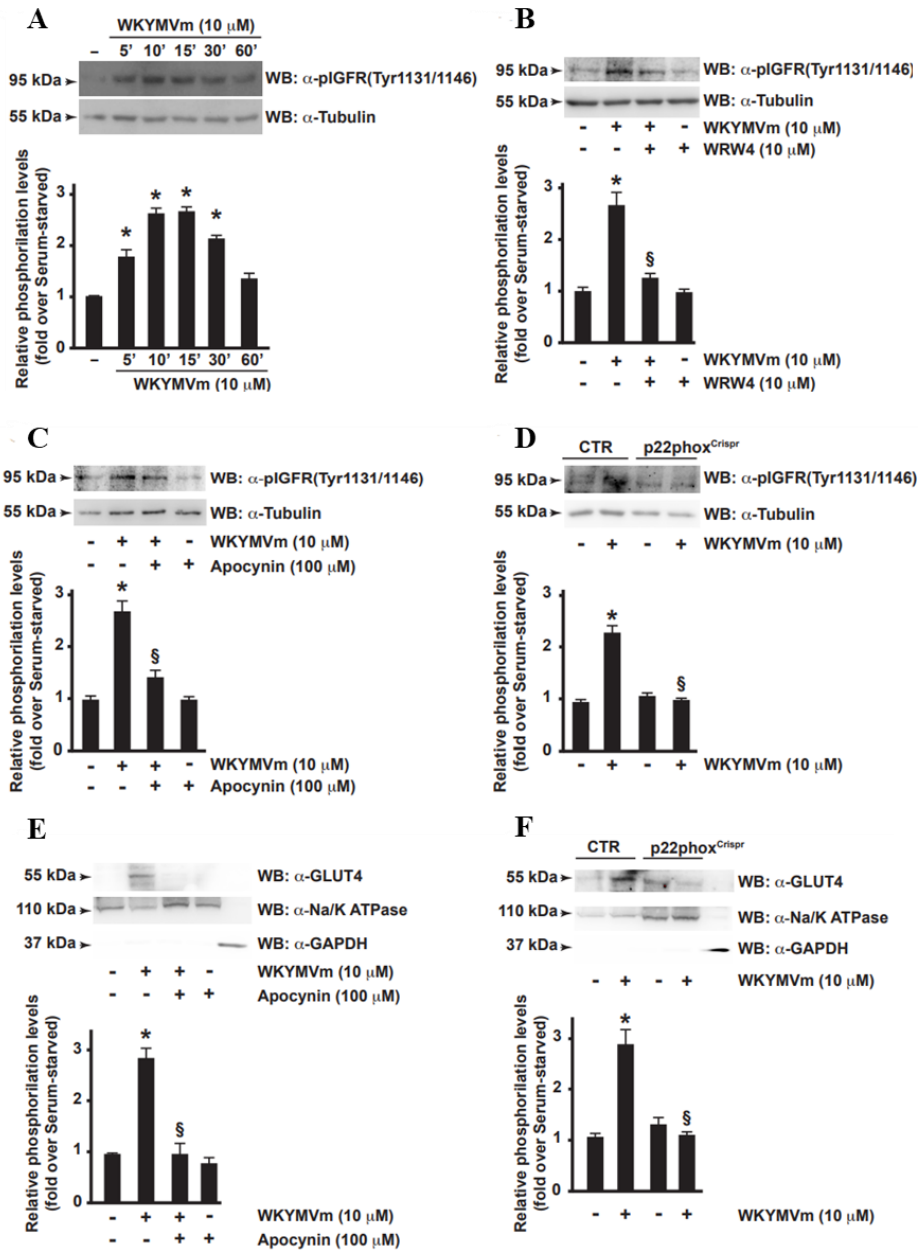

Figure 8. NOX-dependent ROS generation is required for IGF-IRβ transactivation and GLUT4 translocation. FPR2-dependent IGF-IRβ transactivation necessitates NOX2 activation (A-D). CaLu-6 cells were serum starved for 24h and then they are stimulated with WKYMVm for 5,10,15,30,60 min (A) or pretreated with 10μM WRW4 (B), or preincubated with apocynin (C), before stimulation. p22phox$^{Crispr/Cas9}$ were serum depleted for 24h and then stimulated with FPR2 agonist (D). Fifty micrograms of all lysates were resolved on 10% SDS-PAGE and incubated with an anti-pIGF-IR (Tyr1131/1146). An anti-tubulin antibody (α-tubulin) was utilized as a control for protein loading. GLUT4 translocation required ROS generation (E,F). Sixty micrograms of membrane lysates was incubated with an antibody anti-GLUT4 (α-GLUT4). An anti-Na^+/K^+ ATPase antibody was used as a control for protein loading. Cytosolic proteins were loaded and incubated with an anti-GAPDH (α-GAPDH) used as a control. Data represent five independent experiments. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ compared to WKYMVm-stimulated cells.

5.3 FPR2 promotes glucose oxidation via glycolysis

To fulfill the bioenergetic needs of rapid cell division, cancer cells increase glucose uptake and metabolism via glycolysis (DeBerardinis & Chandel, 2016). Glycolysis is regulated at different steps by various mechanisms, but the key control point of the process is the irreversible reaction catalyzed by the 6-phosphofructo-1-kinase (PFK1) enzyme that transforms fructose 6-phosphate (F6P) to F1,6BP. In our metabolomic analysis we observed a significant increase of F1,6BP in FPR2-stimulated cells (**Figure 7A**). PFK1 is regulated by fructose-2,6-bisphosphate (F2,6BP), a key modulator of glycolysis, and by a variety of other metabolites. The bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate (PFKBP2) enzyme regulates intracellular F2,6BP levels. PFKBP2 is characterized by both kinase activity, which converts F6P to F2,6BP, and phosphatase activity, which catalyzes the hydrolysis of a phosphate from F2,6BP to obtain F6P. PFKBP has four different isoforms (PFKBP1-4) that have different activity (Ozcan et al., 2020) (Lee et al., 2020). PFKBP2 is mostly expressed in the lung, brain, and heart (Lee et al., 2020; Minchenko et al., 2003), and its phosphorylation induces an increase in F2,6BP concentration and hence to an enhanced glycolysis (Rider et al., 2004). In human, Ser466 and Ser483 are two main phosphorylation sites of PFKBP2 (Rider et al., 2004). We evaluated PFKBP2 phosphorylation in FPR2-stimulated cells and, by using a specific anti-phospho antibody, we observed that WKYMVm and ANXA1 significantly enhance PFKBP2 phosphorylation at Ser483 in a time-dependent manner (**Figure 9A and C**). This effect was completely prevented by WRW4 preatreatment (**Figure 9B and D**). To note, PFKBP2 Ser483 phosphorylation appears to be sustained for longer times in cells treated with WKYMVm compared to ANXA1. This difference could be due to the diverse nature of the ligands and to the specific domains they recognize on the receptor. Since Ser483 residue of PFKBP2 is a target of Akt (Rider et al., 2004) (Mouton et al., 2010), we pretreated cells with wortmannin or LY294002 before WKYMVm stimulation and we observed that these pretreatments inhibited FPR2-induced PFKBP2 activation (**Figure 9E**). FPR2 signaling triggers Akt activation but to evaluate if other cell surface receptors, besides FPR2, are involved in PI3K/Akt signaling and hence, in PFKBP2 phosphorylation, we preincubated cells with GSK1904529A, an IGF-IR inhibitor (Sabbatini et al., 2009), or LY2874455 a potent selective inhibitor pan-FGFR (Michael et al., 2017), or AG1478, a specific EGFR inhibitor, before WKYMVm stimulation. Interestingly, only FGFR inhibitor prevented PFKBP2 phosphorylation (**Figure 9F**), thus indicating a crosstalk between FPR2 and FGFR in CaLu-6 cells. FGFR1-4 are members of the FGFR family, which belongs to RTKs (Eswarakumar et al., 2005) (Ornitz & Itoh, 2015). The binding of a ligand on FGFRs induces the phosphorylation on Tyr196, Tyr306, Tyr349, Tyr392 and Tyr436 residues of the adaptor/scaffold phosphoprotein FGF receptor substrate 2 (FRS2) (Kouhara et al., 1997) (Hadari et al., 1998) and subsequent activation of PI3K/Akt pathway (Zhang et al., 2013). Western blot analysis demonstrated a time-dependent increase of FRS2 phosphorylation on Tyr436 residue (**Figure 9G**), which was prevented by WRW4 (**Figure 9H**). These findings demonstrate that FPR2 signaling

leads cells to the glycolytic pathway by increasing kinase activity of the bifunctional enzyme PFKBP2 through FGFR/FRS2- and Akt-dependent phosphorylation.

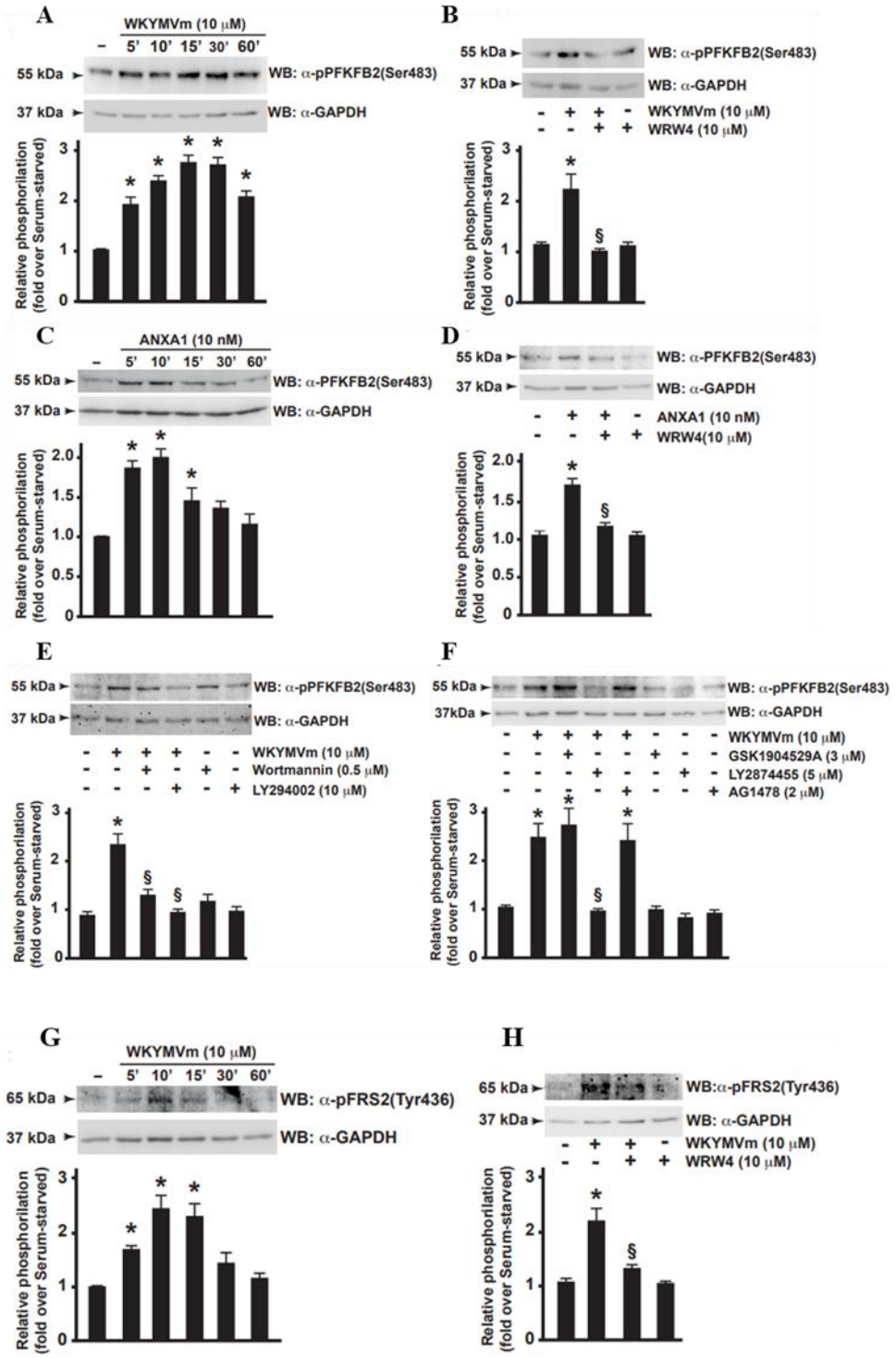

Figure 9. FPR2 signaling triggers glucose oxidation via glycolysis. FPR2 stimulation induces PFKBP2 activation (A). CaLu-6 cells were serum starved for 24 h and stimulated for 5,10,15,30,60 min with WKYMVm (A) or ANXA1 (C). Cells were pretreated with WRW4 before stimulation (B,D). PFKBP2 phosphorylation is due to Akt activation and FGFR1 transactivation (E-H). Cells were stimulated with

10μM WKYMVm, or pretreated with wortmannin, or LY294002 (E), or with GSK1904529A, LY2874455,AG1478 (F), before stimulation. FPR2 signaling activates scaffold protein FRS2 (G,H). CaLu-6 cells were serum depleted for 24 h and then stimulated with WKYMVm (G) or preincubated with FPR2 antagonist (H) before stimulation. Lysates were loaded on 10% SDS-PAGE and immunoblotted with an anti-pPFKBP2(Ser483) antibody (α-pPFKBP2(Ser483)) (A-F), or with an anti-pFRS2(Tyr436) antibody (α-pFRS2(Tyr436)) (G,H). An anti-GAPDH antibody (α-GAPDH) was a control for protein loading. The data represent four independent experiments. *$p<0.05$ compared to unstimulated cells. $^{§}p<0.05$ compared to WKYMVm-stimulated cells.

5.4 FPR2 signaling inhibits the entrance of pyruvate in the tricarboxylic acid cycle

Pyruvate Dehydrogrenase Complex (PDC) is the centre of the aerobic metabolism of carbohydrates. It catalyzes the conversion of pyruvate into Acetyl-CoA, modulating the entry of glucose-derived carbons into the TCA cycle and so regulating energy flow in mammalian cells (Roche & Hiromasa, 2007). PDC consists of three catalytic enzymes and their respective regulatory proteins (Hiromasa et al., 2004). PDC is regulated by reversible phosphorylation-dephosphorylation cycle and hence by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP) (McFate et al., 2008). Pyruvate dehydrogenase is a heterotetrameric enzyme with two alpha (PDHA1) and two beta subunits (PDHB1). PDHK1-4 phosphorylate and inactivate PDHA1, whereas PDP1 and PDP2 dephosphorylate and activate PDHA1. PDHA1 has three main phosphorylation sites: Ser232, Ser293 and Ser300 residues that reduce PDC activity and hence, contribute to metabolic reprogramming of cancer cells by inducing glycolysis and inhibiting the entry of Acetyl-CoA in the TCA cycle (McFate et al., 2008) (Hitosugi et al., 2011) (Shan et al., 2014) (Luo et al., 2019). We observed that WKYMVm or ANXA1 stimulation significantly enhanced PDHA1 phosphorylation on Ser293 (**Figure 10A and C**), and this event was prevented by pretreatment with WRW4 (**Figure 10B and D**). PI3K signaling pathway modulates glucose metabolism and it is also responsible for PDHK1 activation by inducing the phosphorylation on its Thr346 (Chae et al., 2016). Active PDHK1 phosphorylates and inactivates PDC. We preincubated cells with wortmannin or LY29400, before FPR2 stimulation, and we observed that these pretreatments prevented FPR2-mediated PDHA1 phosphorylation on Ser293 (**Figure 10E**). Since PI3K signaling is activated by cytokines, growth factors and insulin, we preincubated cells with specific inhibitors of IGF-IRβ/IRβ, FGFR, and EGFR before WKYMVm stimulation. Western blot analysis showed a significant reduction in PDHA1 phosphorylation on Ser293 only in GSK1904529A pretreated cells (**Figure 10F**), strongly suggesting that PI3K activation is due to insulin receptors. To note, FPR2 induces NOX-dependent ROS generation which in turn can modulate Akt activation and MAPK signaling pathway as well as the activity of several redox-sensitive transcription factors. Therefore, we analyzed the role of NOX in PDHA1 phosphorylation and we observed in both apocynin-preincubated cells (**Figure 10G**) and in p22phox$^{Crispr/Cas9}$ cells (**Figure 10H**) a significative reduction of PDHA1 phosphorylation on Ser293. These results indicate that FPR2 triggers PDC inactivation in IGF-IRβ/IRβ, PI3K and NOX-dependent manner, promoting in this way glycolytic pathway for energy production.

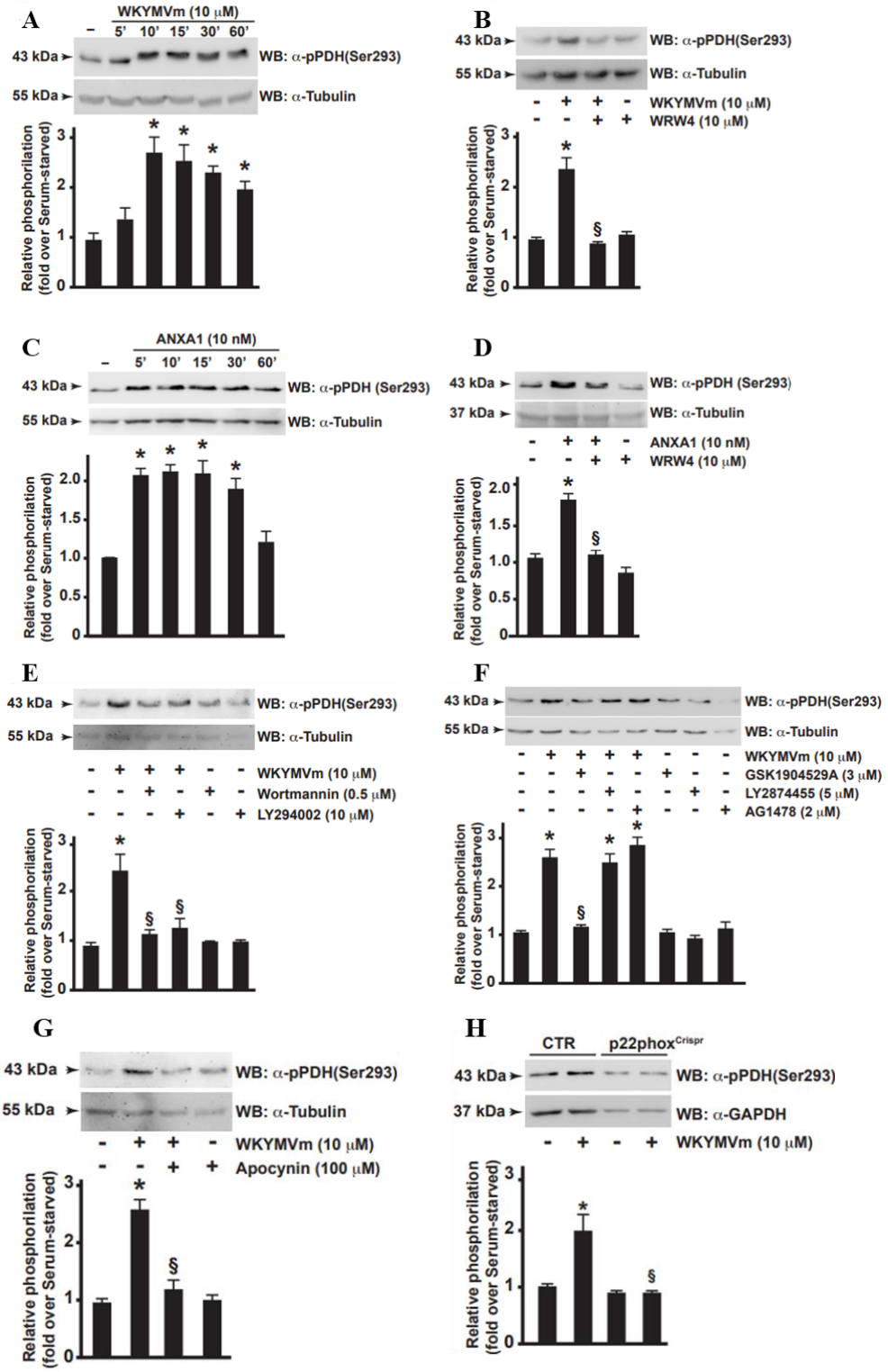

Figure 10. FPR2 signaling inhibits PDH activity.

FPR2 stimulation enhanced PDH phosphorylation in a time-dependent manner (A,C). Serum-deprived CaLu-6 cells were stimulated with WKYMVm (A) or ANXA1(C) for the indicated times. Cells were pretreated with WRW4 before FPR2 stimulation (B,D). PDH phosphorylation is prevented by

wortamannin or LY29400 pretreatments (E). FPR2- mediated PDH phosphorylation required IGF-IRβ (F). Cells were preincubated with GSK1904529A, LY2874455, AG1478 before WKYMVm stimulation (F). PDH phosphorylation depends on NOX activation (G,H). Cells were pretreated with the indicated concentration of apocynin (G) or p22phox$^{Crispr/Cas9}$ were stimulated with WKYMVm (H). The whole lysates were resolved on 10% SDS-PAGE and incubated with an anti-PDH(Ser293) antibody (α-PDH(Ser293)). An anti-GAPDH antibody was utilized as a control for protein loading. Data represent three independent experiments.*$p<0.05$ compared to unstimulated cells. $^{§}p<0.05$ compared to WKYMVm- stimulated cells.

5.5 FPR2 signaling promotes lactate dehydrogenase activity and enhances lactate concentration

Lactate dehydrogenase-A (LDH-A) converts pyruvate into lactate and produces NAD^+ useful to maintain aerobic glycolysis (Bui & Thompson, 2006). LDH-A is regulated by several post-translational modifications such as phosphorylation at its Tyr10 residue that correlates with an enhanced activity of the enzyme (Fan et al., 2011). By western blot analysis, we observed a significant increase of LDH-A phosphorylation at Tyr10 residue in a time-dependent manner, in WKYMVm- and ANXA1-stimulated CaLu-6 cells (**Figure 11A and C**). This increase is prevented by WRW4 pretreatment (**Figure 11 B and D**). The oncogenic receptor tyrosine kinase FGFR1, phosphorylates LDH-A at the Tyr10 residue, inducing the formation of tetrameric LDH-A complex (Fan et al., 2011) (Liu et al., 2018). Since we demonstrated that FPR2 transactivated FGFR, we evaluated the role of FGFR in LDH-A activation in FPR2-stimulated cells. By immunoblot experiments, we observed that WKYMVm-induced LDH-A phosphorylation at Tyr10 residue was prevented by preincubation with LY2874455, the pan-FGFR inhibitor (**Figure 11E**). However, also other tyrosine kinases are involved in Tyr10 phosphorylation of LDH-A, including Src. Therefore, we pretreated cells with PP2, an ATP-competitive inhibitor of the Src protein tyrosine kinase family, or with PP3, a negative control for PP2, and we found that Src inhibitor significantly prevented LDH-A phosphorylation at Tyr10 residue (**Figure 11F**). Src can be recruited on FGFR1 through FRS2 at the plasma membrane (Ren et al., 2011; Sandilands et al., 2007). Src activity is regulated by phosphorylation at Tyr416 residue in its kinase domain. Therefore, we analyzed Src phosphorylation in WKYMVm-stimulated cells preincubated or not with pan-FGFR inhibitor. By using a specific phospho antibody we observed that LY2874455 prevents Tyr416 phosphorylation of Src (**Figure 11G**). In addition, we found that FPR2-dependent LDH-A activation is also responsible for the enhanced lactate generation in FPR2-stimulated cells (**Figure 11H**). Taken together, these data demonstrate that in CaLu-6 cells FPR2 signaling promotes LDH-A activity in FGR1- and Src-dependent manner, thereby increasing lactate production in CaLu-6 cells.

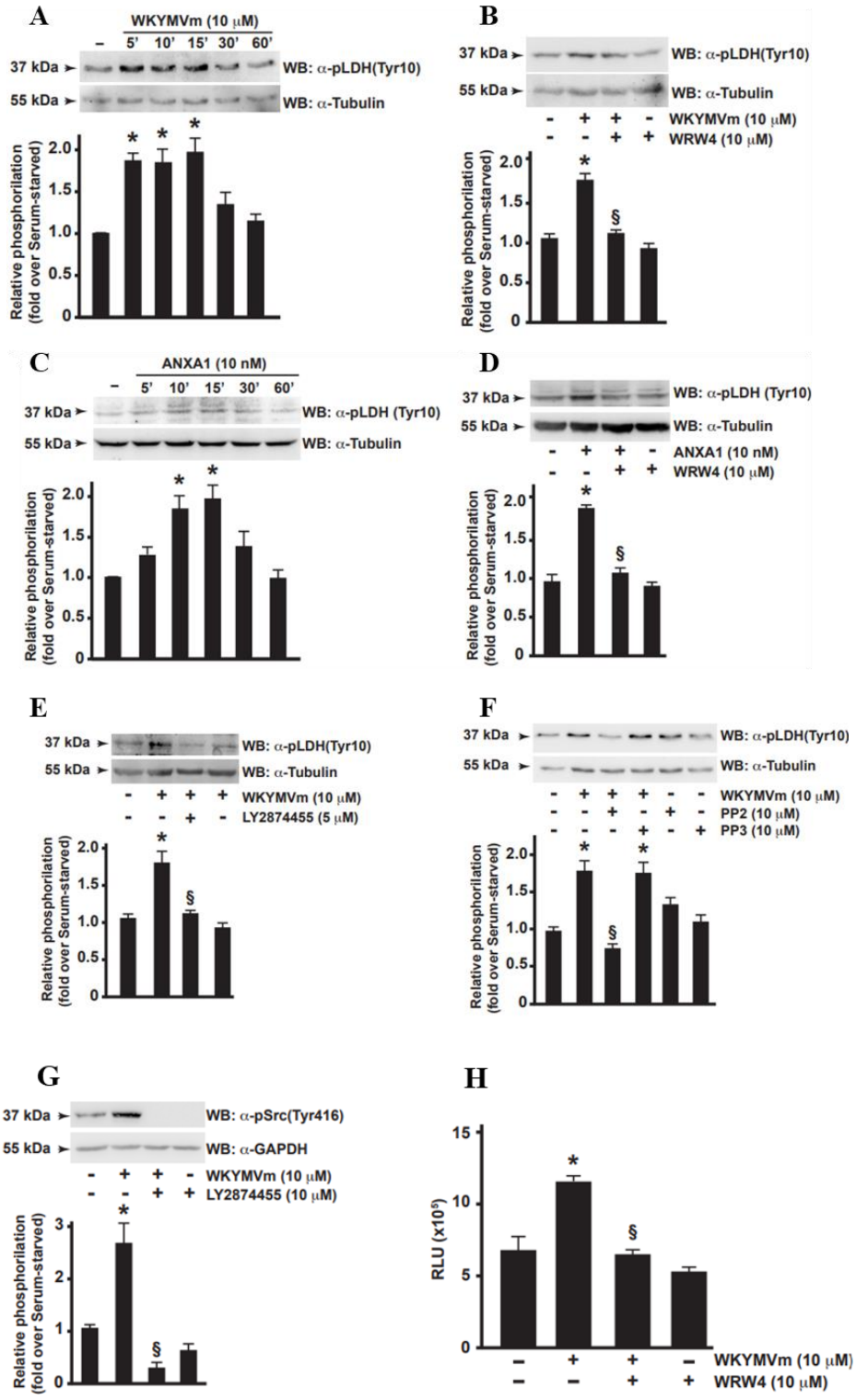

Figure 11. FPR2 stimulation enhances lactate production by increasing LDH activity.
FPR2 stimulation increases LDH phosphorylation in a time-dependent manner (A-D). CaLu-6 cells, after 24h of starvation, were stimulated for 5,10,15, 30 and 60 min with WKYMVm (A) or ANXA1 (C), or

pretreated with WRW4 (B,D). FPR2-dependent FGFR1 transactivation is required for LDH activity (E). Serum-depleted cells were preincubated with LY2874455, a pan-FGFR inhibitor, at the indicated concentration, before WKYMVm stimulation (E,G). Src is recruited to the FGFR1 on the plasma membrane and phosphorylates LDH (E-G). Cells were pretreated with PP2 or PP3 (F), or with LY2874455 (E,G), for the indicated times. Fifty micrograms of the lysates was resolved on 10% SDS-PAGE and incubated with an anti-pLDH (Tyr10) antibody (α-pLDH(Tyr10)) (A-F), or with anti-pSrc (α-pSrc(Tyr416))(G). An anti-GAPDH antibody (α-GAPDH) is a control for protein loading. Data represent five indipendent experiments. Bar graphs depicting lactate concentrations recorded in cell culture medium (H). CaLu-6 cells were serum-starved for 24 h before being stimulated with WKYMVm. Cell culture medium were collected, and lactate content was determined using a commercial kit and the manufacturer's instructions. The results represent the mean of three independent studies, and each point was examined in triplicate in each experiment. *$p<0.05$ compared to unstimulated cells. $^{§}p<0.05$ compared to WKYMVm- stimulated cells.

5.6 FPR2 signaling activates HIF-1 and c-Myc

LDH-A expression is controlled by c-Myc and hypoxia inducible factor-1 (HIF1) (Ždralević et al., 2018). These two transcription factors are involved in metabolic reprogramming of cancer cells by improving their metabolic needs and so increasing glucose absorption and its conversion to lactate. HIF-1 is the central axis of the hypoxic signaling, and it consists of two subunits: α-subunit which is regulated by oxygen concentration and β-subunit which is constitutively expressed. Under normoxic conditions, HIF-1α is degraded by proteasoma after hydroxylation on two proline residues by prolyl hydroxylases; whereas in hypoxic conditions HIF-1α is not degraded and it translocates into the nucleus where it binds HIF-1β (Hirota, 2021). Once activated, HIF-1 recognizes the *hypoxia responsive element* (HRE) on target genes, including those involved in glucose uptake, glycolytic enzyme synthesis, lactate generation and secretion (Semenza, 2010). Therefore, we first investigated FPR2 ability to induce HIF-1 stabilization and we observed a time-dependent increase of this protein in immunoblot analysis performed on whole lysates of CaLu-6 cells (**Figure 12A**). Such increase was inhibited by WRW4 (**Figure 12B**). NOX-dependent ROS generation is involved in hypoxic signaling in primary lung cells and, in turn, in HIF-1α stabilization (Dabral et al., 2019). We found that NOX inhibition with apocynin or silencing by CRISPR/Cas9-based p22phox editing reduces HIF-1 accumulation in FPR2-stimulated CaLu-6 cells (**Figure 12C and D**). FPR2 also localizes in the nuclear fraction of CaLu-6 cells and AGS cells, where it triggers c-Jun, ERKs, and c-Myc activation. In response to a growth-stimulatory signal, c-Myc is stabilized by phosphorylation on its Ser62 residue (Seo et al., 2008). Interestingly, we observed an increased phosphorylation of c-Myc on its Ser62 residue in WKYMVm-stimulated CaLu-6 cells in a time-dependent manner (**Figure 12E**), that was prevented by WRW4 pretreatment (**Figure 12F**). These findings show that FPR2 signaling regulates HIF-1 and c-Myc activation, which are important in the transcriptional regulation of genes involved in glucose metabolism.

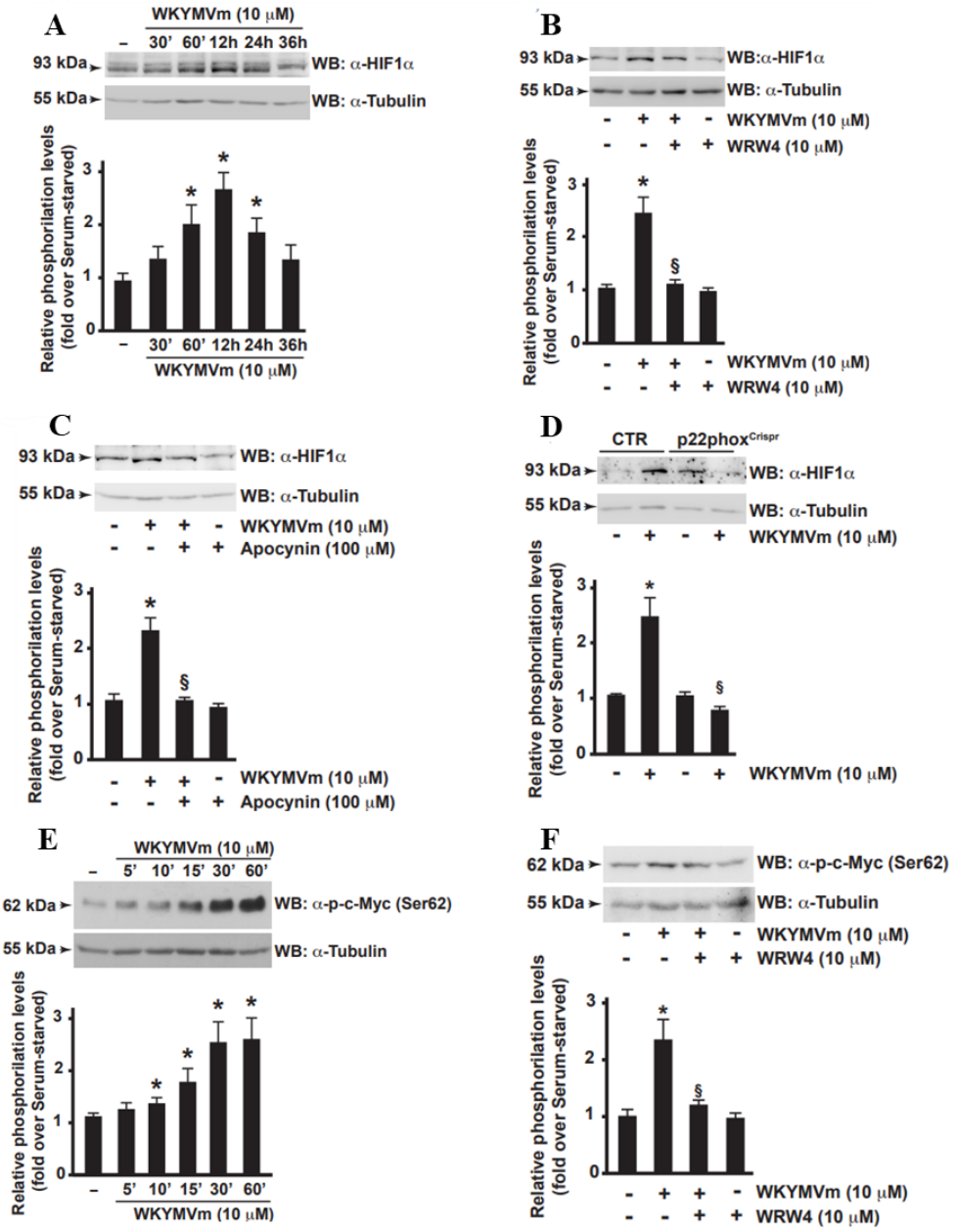

Figure 12. WKYMVm stimulation stabilizes HIF-1 and activate c-Myc.
FPR2 signaling stabilizes HIF-1α in a time-dependent manner (A and B). CaLu-6 cells were serum depleted for 24 h and then they were stimulated with 10μM WKYMVm for the indicated times (A) or pretreated with WRW4 (B). NOX2 activation is required for HIF-1 stabilization (C and D). Cells were preincubated with 100μM apocynin , before WKYMVm stimulation (C). p22phox$^{Crispr/Cas9}$ were serum starved for 24h and then stimulated with WKYMVm for 12h(D). FPR2 signaling induced a time-dependent activation of c-Myc (E). Cells were pretreated with WRW4 before stimulation (F). Fifty micrograms of the total lysates was resolved on 10% SDS-PAGE and incubated with an anti-HIF1α antibody (α-HIF1α) (A-D), or with an anti-p-c-Myc(Ser62) (α-p-c-Myc(Ser62)). An anti-GAPDH was a control for protein loading. Data are representative of five indipendent experiments. *$p<0.05$ compared to unstimulated cells. $^{§}p<0.05$ compared to WKYMVm-stimulated cells.

5.7 FPR2 activation increases CaLu-6 cell energy metabolism

We further investigated on FPR2 effect on glucose metabolism in lung cancer cells by using Seahorse XFA glycolytic assay rate. This assay measures glycolytic rates under basal circumstances and compensatory glycolysis after mitochondrial inhibition. These rates evaluate the contribution of CO2 to extracellular acidification derived from mitochondria or TCA cycle activity and are directly comparable to lactate accumulation data. At the beginning, we measured the real time extracellular acidification rate (ECAR) in serum- starved cells stimulated or not with WKYMVm for 24 h and we observed a significant increase of the ECAR in stimulated cells compared to unstimulated cells (**Figure 13A**). In addition, the proton efflux rate (PER) value calculates the total proton efflux derived from glycolytic and mitochondrial acidification, providing a more accurate measurement of extracellular acidification (pmol H^+ min^{-1}). Interestingly, we found that also PER was significantly enhanced upon WKYMVm stimulation in comparison to unstimulated cells (**Figure 13B**). In order to calculate the glycolytic proton efflux rate (glycoPER), we inhibited mitochondrial respiration by using rotenone and antimycin A (Rot/AA). Our results demonstrated that FPR2 stimulation, measured after mitochondrial electron transport chain blockade, significantly upregulated glycoPER in both basal (**Figure 13C**) and compensatory (**Figure 13D**) glycolysis. Furthermore, WKYMVm stimulation significantly increased mitochondrial basal respiration when compared to untreated CaLu-6 cells, as measured by oxygen consumption rate (OCR) (**Figure 13E and F**). The increased ECAR and OCR in WKYMVm stimulated CaLu-6 cells suggest a switch towards a more energetic phenotype. These results clearly demonstrate that FPR2 stimulation enhances energetic metabolism of CaLu-6 cells (**Figure 13G**).

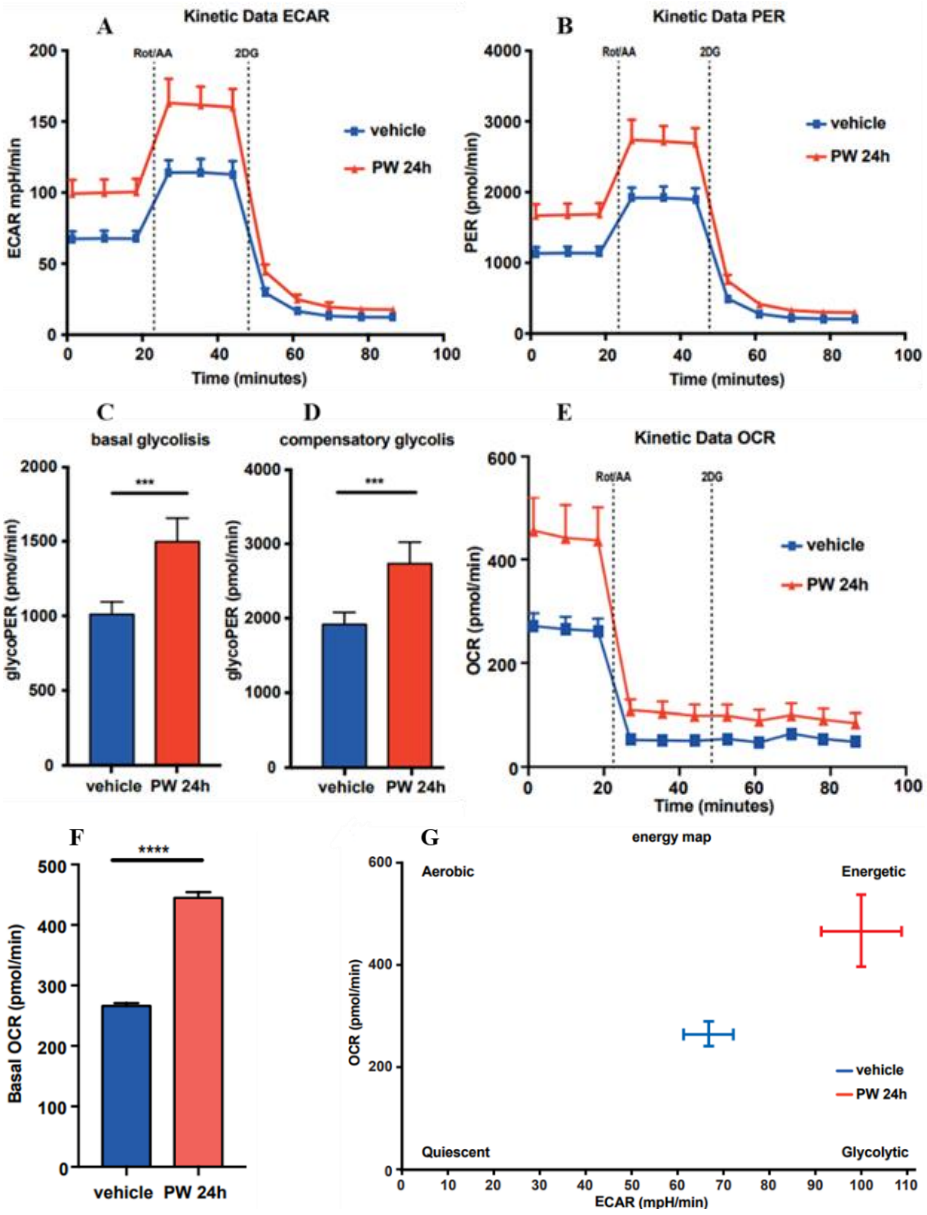

Figure 13. Seahorse analysis of CaLu-6 cells stimulated with WKYMVm peptide.
Extracellular acidification rate (ECAR) and proton efflux rate (PER) were measured in CaLu-6 cells stimulated or with WKYMVm peptide (red line) or vehicle (blu line) for 24 h. Basal ECAR and PER measurements were followed by a sequential treatment (dotted vertical lines) with rotenone plus antimycin A (Rot/AA) and 2-deoxyglucose (2DG). The bar graphs depict baseline (C) and compensatory (D) PER generated by glycolysis (glycoPER). The basal oxygen consumption rate (OCR) of CaLu-6 cells was evaluated after 24 hours of treatment with 10µM WKYMVm (red line) or vehicle (blu line). CaLu-6 cells were exposed for 24h to 10 µM WKYMVm (red) or vehicle (blu) (E, F). The metabolic profile of CaLu-6 cells stimulated with WKYMVm (red) or vehicle (blu) for 24 h (G). ***p<0.001 and ****p<0.0001 in comparison to unstimulated cells.

6.1 FPR2 stimulation promotes Pentose Phosphate Pathway

The metabolomic analysis performed in WKYKVm- stimulated CaLu-6 cells showed also a significant FPR2-dependent increase of ribose 5-phosphate (Ribose-5P), citrate and malate concentration (**Figure 14A**). These data are consistent with the glucose oxidation via Pentose Phosphate Pathway (PPP) and TCA, other than glycolysis. The PPP consists of both oxidative phase and non-oxidative phase. The oxidative phase includes three irreversible reactions. In the first one G6P is converted into to 6-phosphogluconlactone by G6P dehydrogenase (G6PDH) that requires $NADP^+$ as cofactor and thus produces NADPH. Then 6-phosphogluconlactone is subsequently hydrolyzed by phosphogluconolactonase into 6-phosphogluconate. The third irreversible reaction is the oxidative decarboxylation of 6-phosphogluconate to obtain a second molecule of NADPH and ribulose-5-phosphate, which is then converted into ribose-5-phosphate (R5P), a key precursor for nucleotide biosynthesis. NAPDH is useful for reductive biosynthesis and to maintain redox balance under stress situations when cells proliferate rapidly, whereas pentose phosphates are used to supply the high rate of nucleic acid synthesis (Patra & Hay, 2014). Accordingly, our metabolomic investigation in FPR2-stimulated CaLu-6 cells showed increased amounts of AMP, CMP and UMP, which were inhibited by preincubation with WRW4 (**Figure 14B**).

The pyrimidine rings of nucleotides are generated de novo as uracil from aspartate, CO_2 and glutamine. Aspartate is responsible of three of four carbon atoms and one nitrogen atom. The fourth carbon atom comes from CO_2, and the second nitrogen atom comes from glutamine. Five carbon atoms derive from CO2, glycine, and one carbon unit of N10-formyl-TetraHydroFolate (THF), which is derived from the serine-glycine pathway via N5,N10-methylene-THF. As regards, the synthesis of purine rings of nucleotides, five carbon atoms come from CO_2, glycine, and from one carbon unit of N10-formyl-TetraHydroFolate (THF), which derived from the serine-glycine pathway via N5,N10-methylene-THF. Aspartate is an important donor of one nitrogen atom, whereas serine and glycine can be synthesized from 3-phosphoglicerate via de novo synthesis (Lane & Fan, 2015). Notably, in WKYMVm-stimulated CaLu-6 cells, our metabolomic study indicated an FPR2-dependent increases in aspartate, glutamate, and glutamine concentrations (**Figure 14C**).

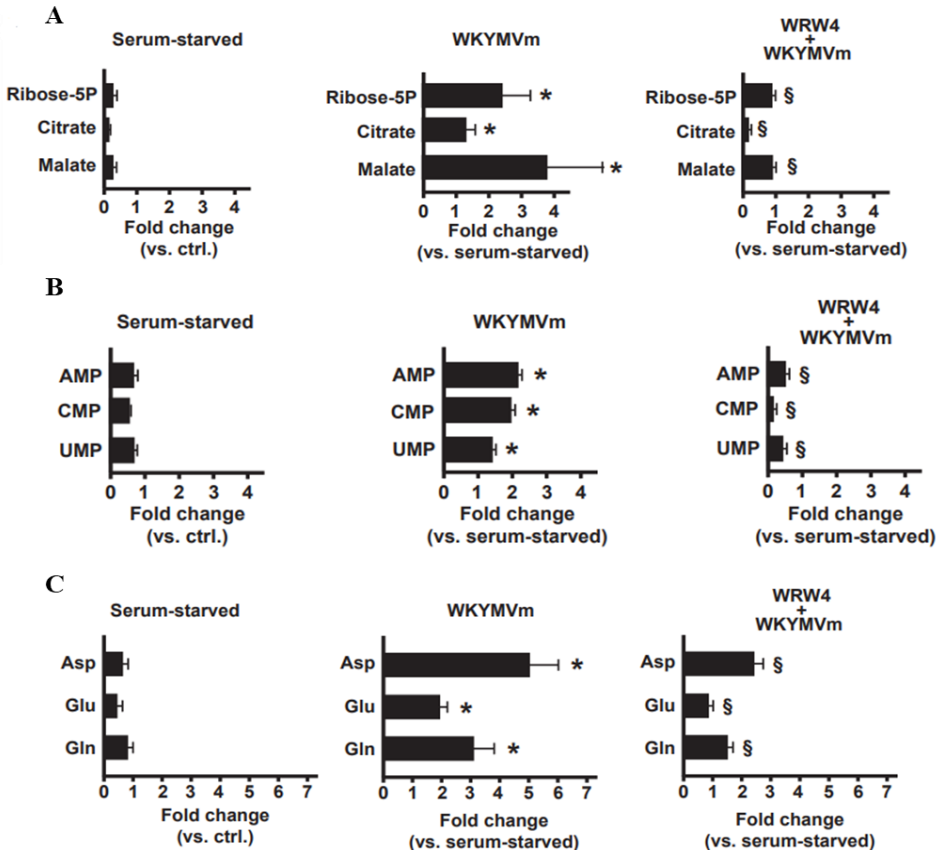

Figure 14. FPR2 stimulation promotes pentose phosphate pathway and nucleotide biosynthesis. CaLu-6 cells were serum starved for 24h and then stimulated or not with 10µM WKYMVm peptide in presence or absence of WRW4. FPR2 signaling modulates glucose metabolism (A), nucleotide biosynthesis (B) and amino acid metabolism (C). *$p<0.05$ in comparison to unstimulated cells. §$p<0.05$ in comparison to stimulated cells. R5P: ribose-5-phosphate; Asp: aspartate; Glu: glutamate; Gln: glutamine.

6.2 FPR2 regulates NADPH production and the non-oxidative phase of PPP

In proliferating cells, PPP is the major source of NADPH. Therefore, we measured NADPH production in WKYMVm or ANXA1 stimulated CaLu-6 cells, and we observed a significant increase of NADPH concentration in a time-dependent manner (**Figure 15A and C**). This increase was prevented by WRW4 (**Figure 15B and D**). Several reports demonstrate that WKYMVm and ANXA1 induce NADPH oxidase-dependent ROS generation in neutrophils and other cell types (Ammendola et al., 2021) (Cattaneo et al., 2011) (Christophe et al., 2001) (Leoni et al., 2013) (Esposito & Carsana, 2019), that depends on a constant source of intracellular NADPH. During oxidative burst, neutrophils shift their metabolism from a glycolysis-dominant metabolism to oxidative PPP, maximizing NADPH generation to fuel superoxide production via NADPH oxidase. Cancer cells are characterized by higher levels of ROS in comparison to normal cells and this increase can enhance the rate of pro-oncogenic mutations and facilitate pro-tumorigenic signaling cascades and may also make cancer cells more vulnerable to energetic and oxidative stress. Thus, the oxidative PPP is required in cancer cells to create high quantities of NADPH to counteract ROS. Overall, the PPP is dependent on glucose availability, and when it is insufficient, a decrease in NADPH concentration may enhance intracellular ROS generation. As a result, other glucose-independent processes for NADPH generation are activated. In our metabolomic study we observed an high level of malate concentration in FPR2-stimulated cells. Interestingly, malate, by exiting the TCA cycle, can produce pyruvate and NADPH in a process catalyzed by the malic enzyme.

The non-oxidative PPP is composed by many reversible reactions that recruit glycolytic intermediates capable of being converted into pentose phosphates and vice versa. These reactions depends on transketolases (TKT) and transaldolases (TALDO), which catalyze reversible reactions and, in turn, can determine the direction of metabolite flux in the non-oxidative PPP. Cancer cells can enhance non-oxidative PPP by increasing TKT and TALDO expression (Patra & Hay, 2014) (Liu et al., 2010). When the metabolic need for nucleotides exceeds that of NADPH, TKT and TALDO divert glyceraldehyde-3-phosphate and fructose-6-phosphate from glycolysis to the non-oxidative PPP to generate extra ribonucleotides. Therefore, cancer cells use the non-oxidative PPP to generate ribonucleotides for de novo synthesis of RNA and DNA. Interestingly, we observed that CaLu-6 cells stimulated with either FPR2 ligands showed a significant increase of TKT activity (**Figure 15E and G**) that is prevented by WRW4 pretreatment (**Figure 15F and H**). These findings strongly suggest that FPR2 signaling stimulates non-oxidative PPP in cancer cells to fuel RNA and DNA synthesis.

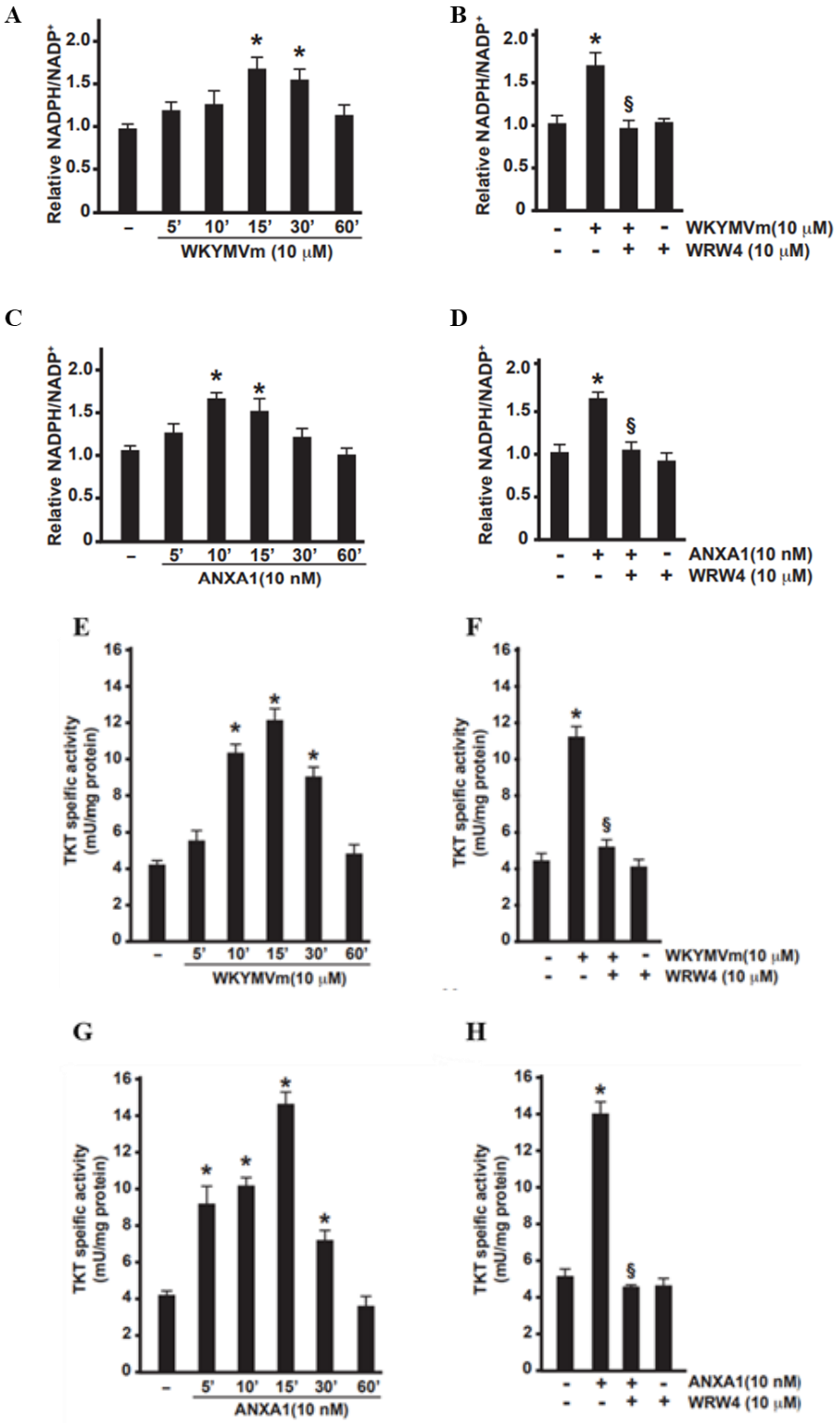

Figure 15. FPR2 stimulation increases NADPH production and modulates the non-oxidative phase of PPP.

CaLu-6 cells were serum depleted for 24h and then stimulated withWKYMVm (A and E) or ANXA1 (C and G). Cells were pretreated with WRW4 before WKYMVm (B and F) or ANXA1 (D and H) stimulation. $NADPH/NADP^+$ assay was performed as reported in Materials and Methods (A-D). The enzyme activity of transketolase (TKT) was evaluated by measuring the fluorescence (RFU) emitted during the conversion of non-fluorescent probe to a fluorescent probe ($\lambda Ex=535/\lambda Em=587$). Bar graphs represent the data (E-H). *$p<0.05$ in comparison to unstimulated cells. § $p<0.05$ in comparison to stimulated cells.

6.3 FPR2 signaling regulates ASCT2 expression

In order to satisfy their energy demand and high rate of protein synthesis, tumor cells require an enhanced demand of amino acids and consequently they reconfigure amino acid metabolism. The presence of particular cell membrane transporters is necessary for amino acid consumption, and several amino acids carriers are over-expressed in various forms of cancer. Some amino acids are used more than others in tumor cells (Scalise et al., 2020). For instance, glutamine is the most abundant amino acid and elevated levels of glutamine provide cancer cells with an available source of carbon and nitrogen atoms to sustain macromolecule synthesis, energy processes, and cellular homeostasis (Altman et al., 2016b). Many membrane proteins, including the well-studied solute carrier family 1 neutral amino acid transporter member 5 (SLC1A5, or known as ASCT2) (Wise & Thompson, 2010), are involved in the transport of glutamine into the cells (Bhutia et al., 2015). Antiporters transport glutamine out of the cell in exchange for other amino acids, such as leucine via LAT1, a heterodimer of SLC7A5 and SLC3A2 (Nicklin et al., 2009). ASCT2 promotes cell proliferation (Marshall et al., 2017) and it is up-regulated in several forms of cancer. Furthermore, its higher expression is associated with higher glutamine absorption (V. M. Wang et al., 2019). Interestingly, we observed that WKYMVm or ANXA1 stimulation significantly enhanced ASCT2 expression in a time dependent manner (**Figure 16A and C**), that was prevented by WRW4 pretreatment (**Figure 16B and D**). The molecular mechanisms that control ASCT2 activity are still unknown. It has been found that ASCT2 is regulated by miR-137 and the endoplasmic reticulum-associated E3 ubiquitin ligase RNF5nd in numerous types of cancer (Dong et al., 2017; Jeon et al., 2015). Furthermore, SLC1A5 is regulated by the discoid protein domain receptor 1, a kind of the transmembrane receptor tyrosine kinase (Pan et al., 2022). To note, c-Myc overexpression triggers glutamine addiction in cancer cells, increasing survival and proliferation. c-Myc is crucially involved in ASCT2 expression, and thus in enhancing glutamine uptake. We observed that WKYMVm (**Figure 12E**) or ANXA1 (**Figure 16E**) stimulation enhance c-Myc phosphorylation on Ser62 in a FPR2-dependent manner (**Figure 12F and 16F**). These findings show that FPR2 signaling participates in the transcriptional regulation of ASCT2 by modulating c-Myc activity.

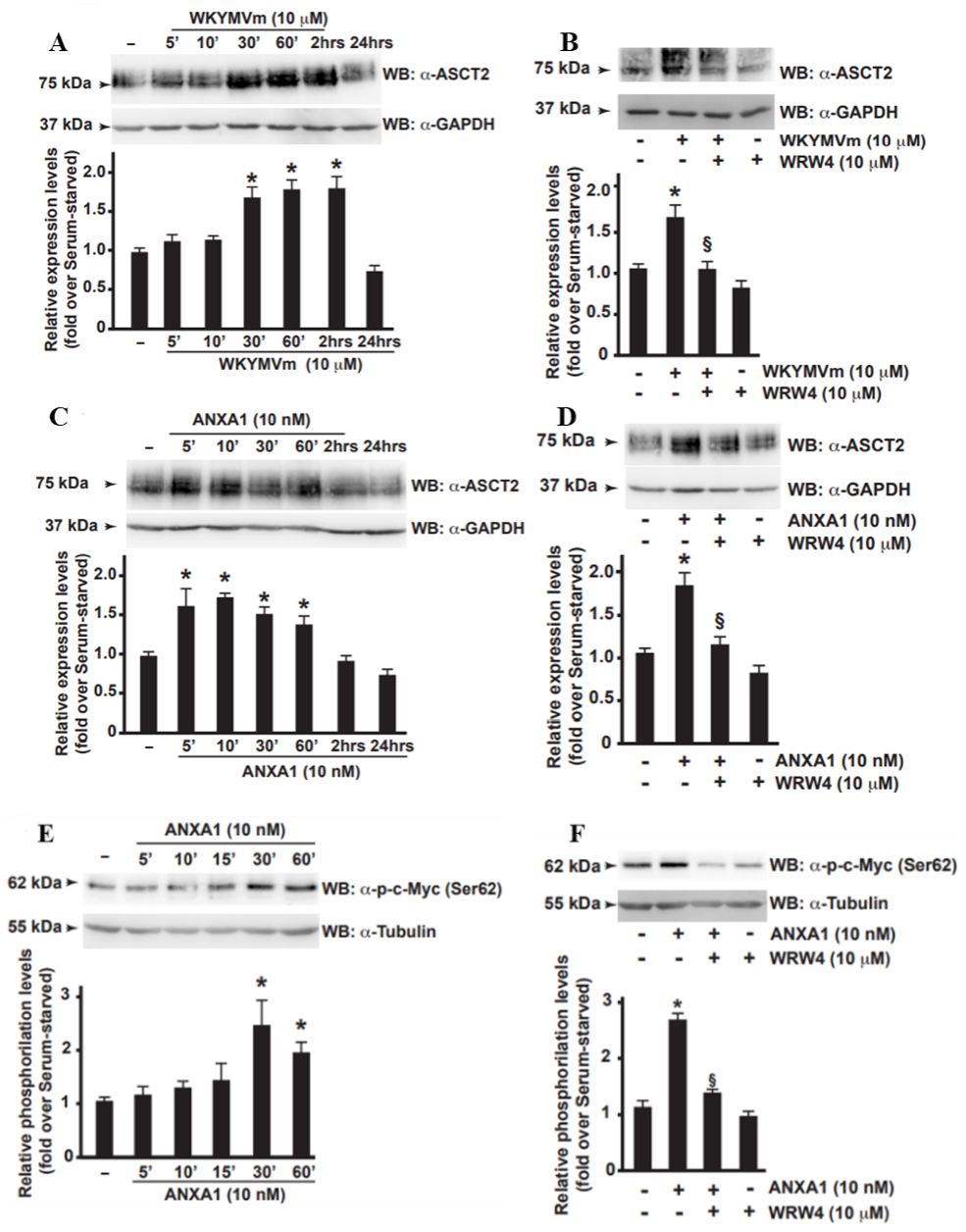

Figure 16. FPR2 stimulation modulates ASCT2 expression through c-Myc activation. CaLu-6 cells were serum depleted for 24h and then stimulated with WKYMVm (A) or ANXA1 (C and E) for the indicated times. Cells were pretreated with WRW4 before WKYMVm (B) or ANXA1 stimulation (D and F). Sixty micrograms of the total lysates were resolved on 10% SDS-PAGE and incubated with anti-ASCT2 (α-ASCT2) for the indicated times. An anti-GAPDH (α-GAPDH) was a control for protein loading. Cells were also incubated with an anti-p-c-Myc (Ser62) (α-p-c-Myc(Ser62)) antibody. An anti-Tubulin (α-Tubulin) antibody was utilized as a control for protein loading. Bar graphs represent three different experiments. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ in comparison to stimulated cells.

6.4 FPR2 signaling modulates the novo synthesis of pyrimidine nucleotides

Our metabolic analysis demonstrated a significant increase of aspartate, glutamine, UMP and CMP concentrations (**Figure 14B and C**). Aspartate participates to the biosynthesis of pyrimidine nucleotides, since it provides three of the four carbon atoms and one nitrogen atom. Glutamine provides the second nitrogen atom. UMP is an intermediate product in the *de novo* synthesis of pyrimidine nucleotides and it can be further phosphorylated to obtain UDP and UTP. CTP synthetase catalyzes the conversion of UTP into CTP in an ATP-dependent reaction where glutamine is an amine donor. Dephosphorylation of CTP results in the formation of CDP and CMP. Otherwise, ribonucleotide reductase can deoxygenate UDP and CDP to form dUDP and dCDP, which can then be phosphorylated (Li et al., 2021). Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) form a multifunctional enzyme that participates in the three initial speed-limiting steps of the de novo synthesis of pyrimidine nucleotides in mammals; it is able to supply all pyrimidine ribonucleotides and deoxyribonucleotides for RNA and DNA biosynthesis. CAD localizes in the cytoplasm of resting cells and translocates to the nucleus once phosphorylated by MAPK on its Thr456 residue as the cell enters S phase. The basal CAD activity is restored by PKA-mediated dephosphorylation at Thr456 and phosphorylation at Ser1406 as cells exit S phase (Sigoillot et al., 2005). In order to stimulate the first three-initial speed limiting steps of the *de novo* synthesis of pyrimidine nucleotides, CAD has to be phosphorylated on Ser1859 by S6 kinase 1 (S6K1), a ribosomal protein target of mTORC1 (Ben-Sahra et al., 2013). Therefore, we evaluated CAD phosphorylation at Ser1859 in FPR2-stimulated CaLu-6 cells, and we observed a significant increase of CAD phosphorylation after WKYMVm as well as ANXA1 stimulation (**Figure 17A and C**). Preincubation with WRW4 prevents this event (**Figure 17B and D**), suggesting that CAD phosphorylation depends on FPR2 activation. S6K1 activity is tightly controlled. Redox-sensitive pathways regulate S6K1 phosphorylation, S6K1-mTORC1 association, and the kinase activity of the S6K1-mTORC1 complex (Sarbassov & Sabatini, 2005). Since FPR2 stimulates NADPH oxidase activation in a variety of cell types (Ammendola et al., 2021; Cattaneo et al., 2011; Cattaneo et al., 2019), we investigated the capacity of NADPH oxidase-dependent ROS generation to modulate CAD phosphorylation at the Ser1859. Therefore, we stimulated with WKYMVm or ANXA1 both apocynin-pretreated CaLu-6 cells and $p22phox^{Crispr/Cas9}$ cells and we don't observed any significant variation in CAD phosphorylation suggesting that this event required NADPH oxidase activation (**Figure 17E-H**).

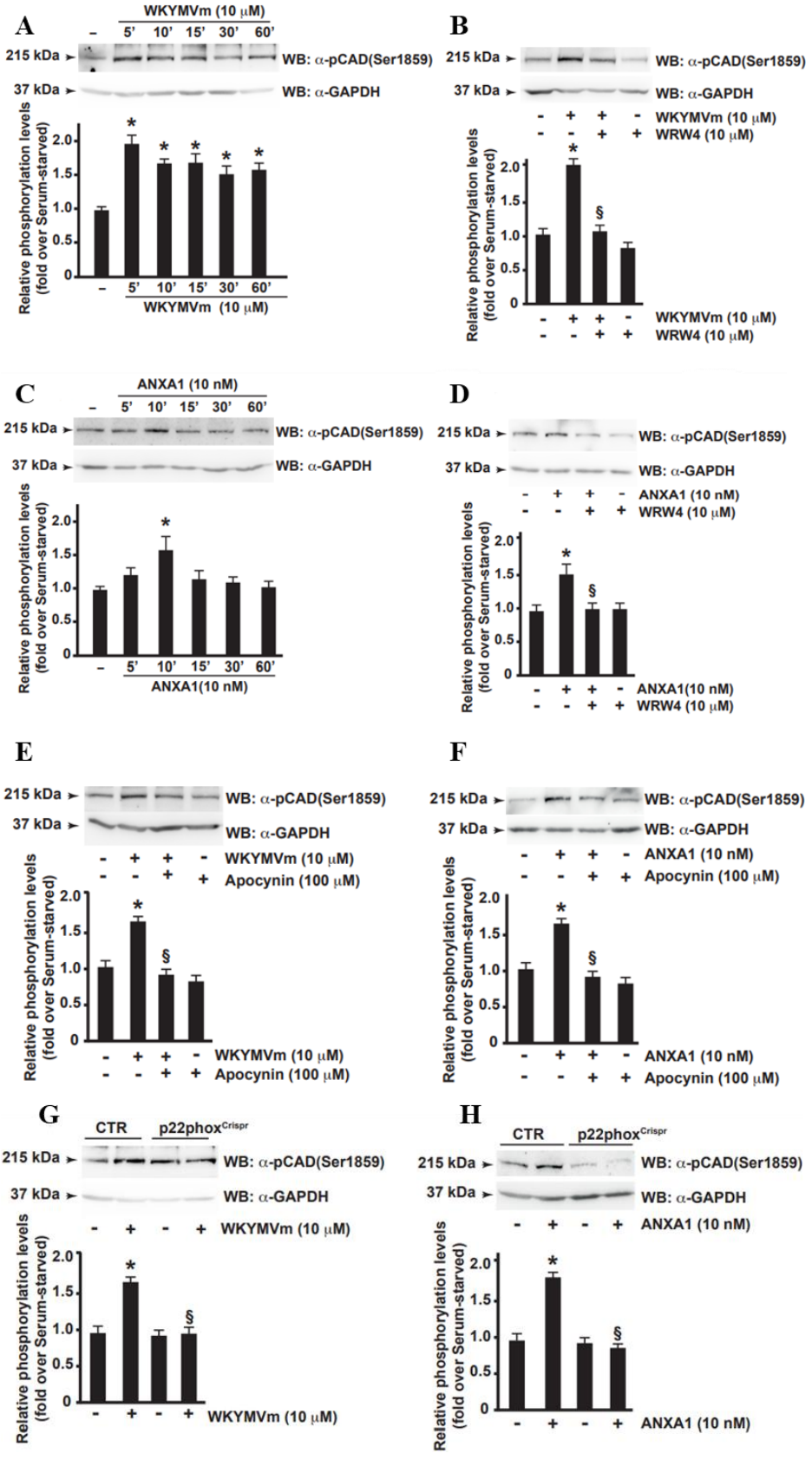
A
WKYMVm (10 μM)
– 5' 10' 15' 30' 60'
215 kDa
WB: α-pCAD(Ser1859)
37 kDa
WB: α-GAPDH
Relative phosphorylation levels (fold over Serum-starved)
WKYMVm (10 μM)
B
WB: α-pCAD(Ser1859)
WB: α-GAPDH
WKYMVm (10 μM)
WRW4 (10 μM)
C
ANXA1 (10 nM)
ANXA1(10 nM)
D
ANXA1 (10 nM)
E
Apocynin (100 μM)
F
G
CTR
p22phoxCrispr
H

Figure 17. FPR2 signaling activates CAD in a NOX2-dependent manner
CaLu-6 cells were serum starved for 24h and then stimulated with WKYMVm (A) or ANXA1(C) for the indicated times. Growth-arrested cells were also preincubated with WRW4 (B and D) or apocynin (E and F). p22phox$^{Crispr/Cas9}$, after 24h of starvation, were stimulated with WKYMVm (G) or ANXA1 (H). Sixty micrograms of the total lysates were resolved on 10% SDS-PAGE and incubated with anti-p-CAD (Ser1859) (α-p-CAD (Ser1859)) antibody. An anti-GAPDH (α-GAPDH) was a control for protein loading. *$p<0.05$ in compariso to unstimulated cells. §$p<0.05$ in comparison to stimulated cells.

6.5 FPR2 signaling controls Tricarboxyl Acid Cycle

In WKYMVm-stimulated cells we observed a significant increase of glutamate concentration (**Figure 14C**). When glutamine enters into the cell via SLC1A5/ASCT2, glutaminases converts glutamine into ammonium and glutamate, which in turn is catabolized through two distinct pathways. In particular, glutamate can be converted into α-ketoglutarate (α-KG) by glutamate dehydrogenase or by aminotransferases. Glutamate dehydrogenase produces as a byproduct $NH4^+$, whereas aminotransferases produce other amino acids. α-KG may feed the TCA cycle, generating ATP through the formation of NADH and FADH2 as well as citrate, which can be used to transport Acetyl-CoA in the cytosol and thus for lipids biosynthesis. Interestingly, we uncovered an increase of citrate concentration in FPR2-stimulated cells (**Figure 14A**). Furthermore, by MitoTracker analysis we observed that WKYMVm stimulation caused an FPR2-dependent and a time-regulated increase of mitochondrial membrane potential (**Figure 18A and B**).

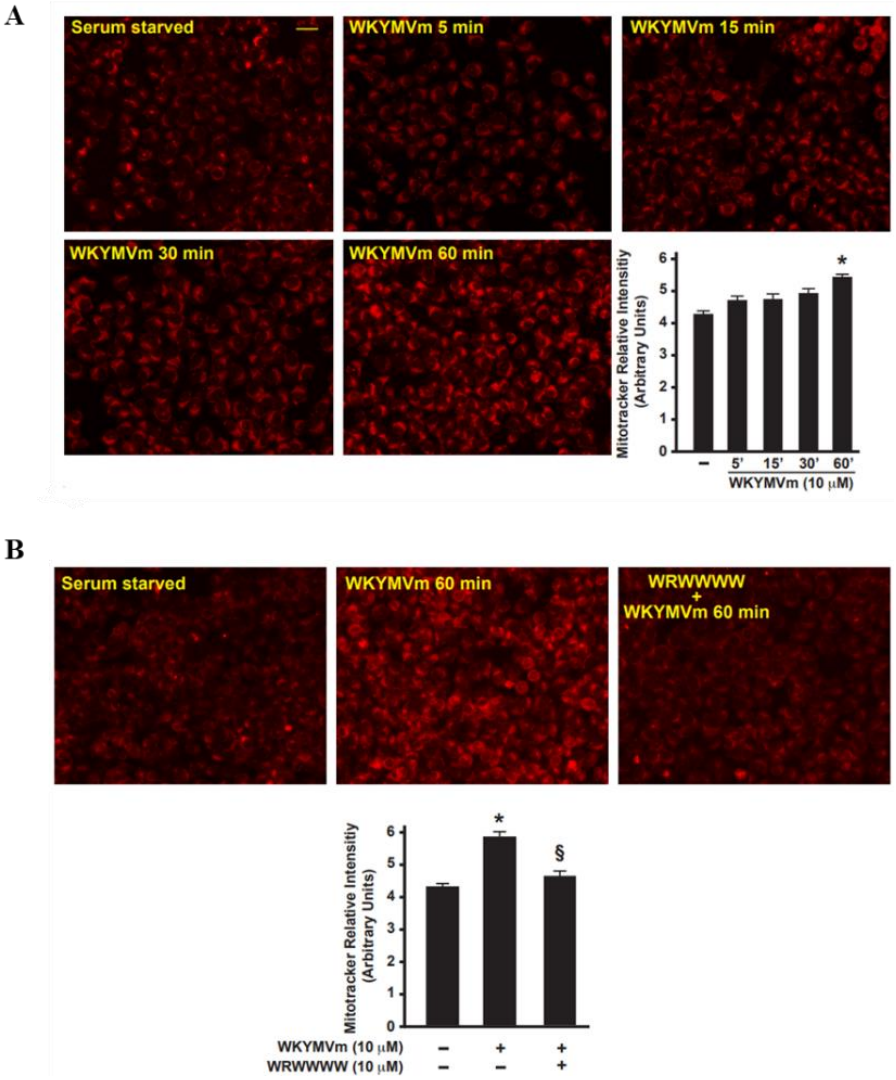

Figure 18. FPR2 signaling triggers changes in mitochondrial membrane potential.
Serum-deprived CaLu-6 cells were stimulated with 10μM WKYMVm for 5, 10, 15, 30, or 60 min (A) and pretreated with WRW4 before stimulation (B). Cells were treated with the probe, and mitochondrial fluorescence was quantified in a Perkin Elmer Envision 2105 Multiplate reader with a built-in monochromator (Perkin Elmer). For normalization, the total number of cells in each well was used. The results represent the means of three independent studies, with each experiment analyzing each experimental point in triplicate. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ in comparison to stimulated cells.

7.1 FPR2 signaling modulates arachidonic acid metabolism

Lipid metabolism is an emerging aspect of metabolic reprogramming. Previously we showed FPR2-mediated effects on aerobic glycolysis, PPP, glutamine transport, and the *de novo* synthesis of pyrimidine nucleotides. Nevertheless, our metabolomic analysis showed a significant increase of arachidonate (AA) concentration in WKYMVm-stimulated CaLu6 cells that was prevented by WRW4 pretreatment (**Figure 19A**). However, stearate, pentadecanoate and laurate level were not affected by FPR2 stimulation. Cells can get AA primarily via three mechanisms: i) direct exogenous uptake via specialized transporters such as CD36 and TWIK-related AA-stimulated K^+ (TRAAK) channels, ii) de novo synthesis from linoleic acid and iii) cPLA2 enzymatic cleavage of the sn-2 position of exiting membrane phospholipids (Hanna & Hafez, 2018) (Koundouros & Poulogiannis, 2020) (Casas et al., 2022). In some cell types, an increase in intracellular Ca^{2+} concentration significantly regulate cPLA2 activity (Kita et al., 2019) (Leslie, 2015); however unlike other PLA2 family members, cPLA2α does not require Ca^{2+} for enzymatic activity (Casas et al., 2022). In addition to Ca^{2+}, intracellular lipids level regulates cPLA2 allosterically activating the enzyme and increasing its residence time in membranes (Stahelin et al., 2007). Moreover, cPLA2 has three different phosphorylation sites. Phosphorylation at Ser515 and Ser727 residues depends on cell type and stimulation conditions, whereas Ser505 phosphorylation is required for cPLA2 activity and its membrane translocation (Pérez-Chacón et al., 2009). We found a time-dependent increase of cPLA2 phosphorylation at Ser505 residue in WKYMVm-stimulated CaLu-6 cells (**Figure 19B**), which was prevented by preincubation with the FPR2 antagonist (**Figure 19C**). FPR2 signaling increases cPLA2 expression in SAA-stimulated cells (Li et al., 2013), as well as in conjunctival goblet cells and neutrophils treated with ANXA1 (Lyngstadaas et al., 2021) or WKYMVm (Bae et al., 2003), respectively. In this study, we showed for the first time that FPR2 signaling activates cPLA2 via phosphorylation at Ser505 residue, thus contributing to the increase of AA concentration observed in the metabolomic analysis.

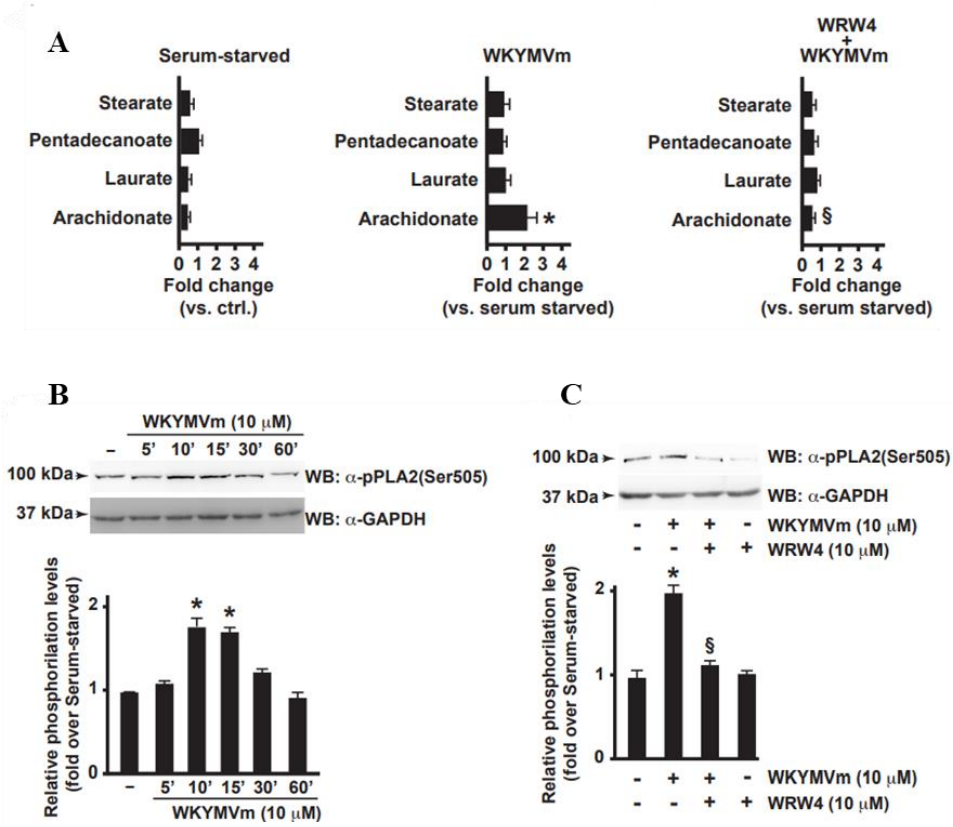

Figure 19. FPR2 stimulation modulates arachidonic acid metabolism.
CaLu-6 cells were serum starved for 24 h and then stimulated or not with 10μM WKYMVm in presence or absence of WRW4 (A). Cells, after 24h of starvation, were treated with WKYMVm for the indicated times (B) and pretreated with WRW4 (C). Sixty micrograms were resolved on 10% SDS-PAGE and incubated with an anti-p-cPLA2 (Ser505) antibody (α-p-cPLA2 (Ser505)) (B and C). An anti-GAPDH antibody (α-GAPDH) was employed as a control for protein loading. Data represent three different experiments. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ in comparison to stimulated cells.

7.2 FPR2 induces cPLA2 phosphorylation in a NOX2-dependent manner

Ser505 residue of cPLA2 is a target of several kinases, including ERKs and p38MAPK (Nito et al., 2008). Furthermore, there is increasing evidence that NOX-dependent ROS generation can induce cPLA2 activation and that hydrolytic product of this enzyme such as AA, could increase NADPH oxidase activity (Pessach et al., 2001) (Cherny et al., 2001) (Zhao et al., 2002). The assembled NOX oxidase complex serves as a target for anchoring cPLA2 to plasma membranes (Shmelzer et al., 2003). The C-terminal domain of gp91phox, in particular, provides an anchoring site for cPLA2 (Pessach et al., 2001). Therefore, it is possible that these two enzymes have a common mechanism for activation by intracellular kinases. ERKs and p38MAPK trigger both cPLA2 phosphorylation and ROS production and both events are prevented by NADPH oxidase inhibitors. Numerous GPCR agonists increase NADPH oxidase-dependent ROS concentrations and elicit the activity of several cytosolic kinases, including p38MAPK, ERKs, and JNK, which can phosphorylate cPLA2. These kinases can also be triggered by the GPCR-dependent TKR transactivation pathways (Daub et al., 1996). We previously established that intracellular domains of active FPR2 promote signaling to G proteins, which activate multiple agonist-dependent signaling cascades, including PLC, PKC isoforms, p38MAPK, PI3K/Akt, and the MAPK pathway. Intracellular signaling cascades triggered by FPR2 also include TKR transactivation, phosphorylation and nuclear translocation of regulatory transcriptional factor, calcium release, and oxidant generation (Cattaneo et al., 2013a). Therefore, we analyze the molecular mechanisms involved in FPR2-induced cPLA2 phosphorylation. Therefore, we pretreated cells with PD098059, a selective inhibitor of MEK, or with SB203580, a p38MAPK inhibitor, before WKYMVm stimulation. Western blot experiments showed that both inhibitors prevent cPLA2 phosphorylation at Ser505 residue (**Figure 20A**). We previously demonstrated that WKYMVm stimulation induces ERK-dependent p47phox phosphorylation and membrane translocation, as well as NADPH-oxidase-dependent superoxide production (Cattaneo et al., 2011). Therefore, we investigated the role of NOX in Ser505 phosphorylation by pretreating cells with apocynin before FPR2 stimulation, or upon stimulation of $p22phox^{Crispr/Cas9}$ cells. In both cases, we observed a significant reduction of cPLA2 phosphorylation at Ser505 residue (**Figure 20B and C**), suggesting that FPR2 modulates cPLA2 phosphorylation in a ERK-, p38MAPK- and NOX-dependent manner.

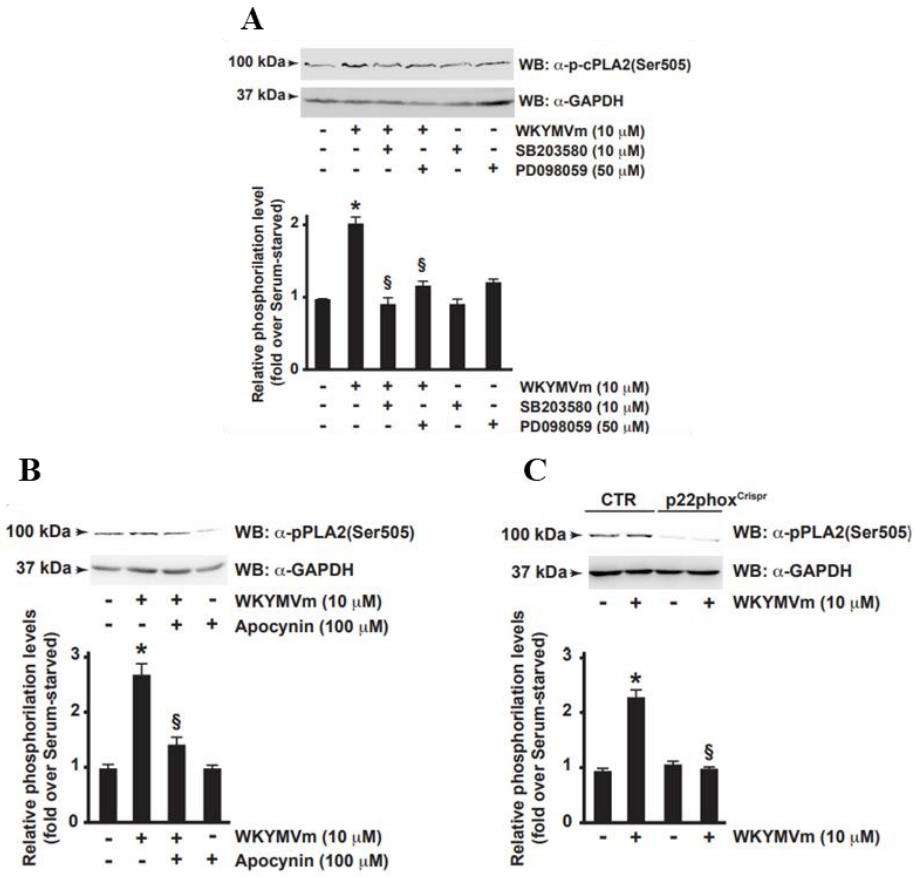

Figure 20. FPR2 triggers cPLA2 phosphorylation in a NOX2-dependent manner.
CaLu-6 cells were serum depleted for 24h and then pretreated with SB203580, or PD098059(A) or apocynin (B) before WKYMVm stimulation. p22phox$^{Crispr/Cas9}$ were serum depleted for 24h and then stimulated with FPR2 agonist (C).Sixty micrograms of the whole lysates were resolved on 10% SDS-PAGE and incubated with an anti-p-cPLA2 (Ser505) antibody (α-p-cPLA2 (Ser505)). An anti-GAPDH antibody (α-GAPDH) was employed as a control for protein loading. Data represent three different experiments. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ in comparison to stimulated cells.

7.3 FPR2 signaling triggers 5-LOX activation

AA generated within the cells can be metabolized by three different enzymes: lipoxygenases (LOX) that produce mainly leuokotrienes (LT), cytochrome p450 and cyclooxygenases that produce prostaglandins (PG). LT belong to a family of lipid mediators with a pivotal role in inflammatory disorders. LTA4 is produced by AA by the sequential action of 5-LOX and FLAP, whereas LTB4 and LTC4 derived from the action of LTA4 or LTC4 synthase, respectively. LTA4 can also be converted into LXA4 by 12-LOX or 15-LOX (Wan et al., 2017). 5-LOX is normally expressed in various leukocyte cell types, but its aberrant expression has been observed in several cancer cells (Hennig et al., 2005; Wang & Dubois, 2010) and, in addition to inflammatory processes, this enzyme is involved in cell differentiation, oxidative stress and in the progression of different diseases (Wang et al., 2017) (Mashima & Okuyama, 2015) (Chen et al., 2023). In resting cells, 5-LOX localizes in the cytosol or in a soluble compartment inside the nucleus, whereas once activated, it translocates into the nucleus where it colocalizes with cPLA2 and FLAP. Nuclear import signals in the 5-LOX sequence determine nuclear import. 5-LOX can also be activated by a variety of soluble and particulate stimuli, as well as ionophores that cause an increase in intracellular Ca^{2+} concentration. Ca^{2+} regulates 5-LOX translocation into the nucleus and its association with membrane by binding the putative N-terminal C2-like domain of 5-LOX (Hammarberg et al., 2000). 5-LOX is a substrate for several kinases, and phosphorylation of different residues has distinct effects on 5-LOX subcellular distribution and activity. Phosphorylation at Ser271 and Ser663 residues enhances 5-LOX synthesis and is strongly promoted by unsaturated fatty acids including AA. Enhanced 5-LOX activity following dual phosphorylation at Ser271 and Ser663 residues, is observed when intracellular Ca^{2+} levels are low and, thus, insufficient to activate 5-LOX alone. Ser271 phosphorylation is responsible of 5-LOX localization in the nucleus (Rådmark et al., 2015), whereas, 5-LOX phosphorylation at Ser523 residue prevents its nuclear translocation (Luo et al., 2005). Ser523 is a target of phosphorylation by PKA (Luo et al., 2004). We investigated the ability of FPR2 stimulation to induce Ser271, Ser663 and Ser523 phosphorylation of 5-LOX. Our results showed that exposure of CaLu6 cells to WKYMVm increases 5-LOX phosphorylation at Ser271 and Ser663 residues, but not at Ser523 residue (data not shown) in a time-dependent manner (**Figure 21A and C**) and sensible to WRW4 pretreatment (**Figure 21B and D**). These results demonstrate that 5-LOX activation depends on FPR2 signaling.

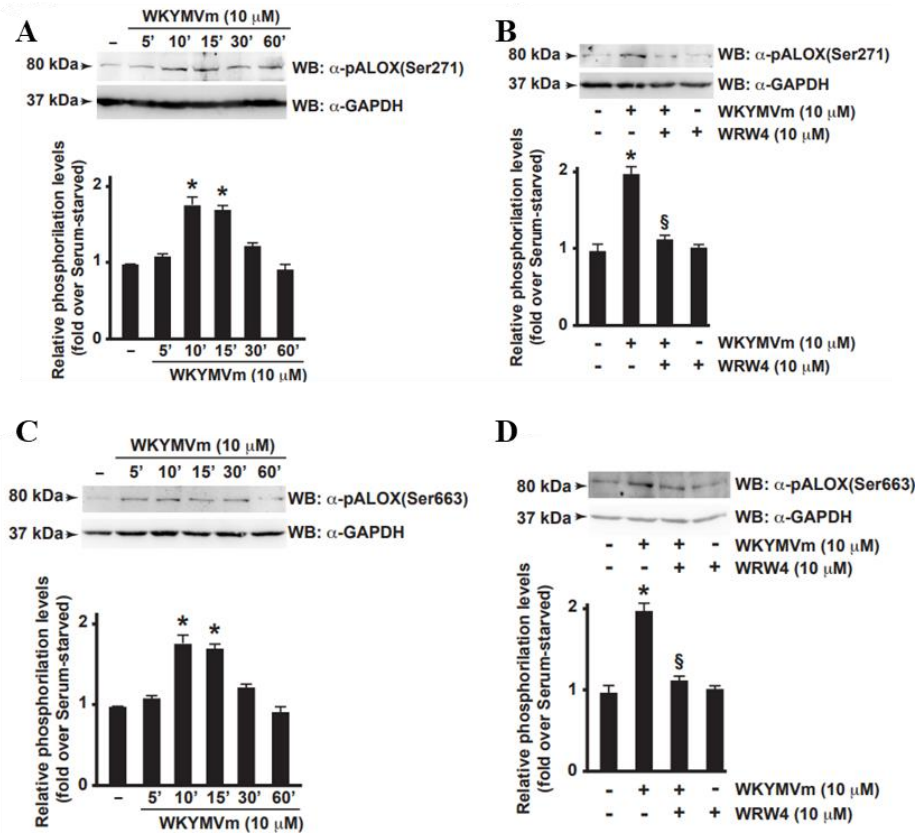

Figure 21. FPR2 signaling triggers 5-LOX activation.
CaLu-6 cells were serum starved for 24 h and then stimulated or not with WKYMVm peptide for the indicated times (A and C) in presence or absence of WRW4 (B and D). Sixty micrograms of the whole lysates were resolved on 10% SDS-PAGE and then incubated with an anti-p-ALOX (Ser271) antibody (α-p-ALOX (Ser271) (A and B) or with an anti-p-ALOX (Ser663) antibody (α-p-ALOX (Ser663)) (C and D). An anti-GAPDH was utilized as a control for protein loading. Bar graphs represent three different experiments. *$p<0.05$ in comparison to unstimulated cells. $^{§}p<0.05$ in comparison to stimulated cells.

7.4 FPR2 signaling induces 5-LOX activation in a p38MAPK, ERKs and NOX2-dependent manner

Ser271 residue of 5-LOX is a target of p38MAPK (Sun et al., 2019), whereas Ser663 residue is a target of ERKs. Dual phosphorylation by ERK2 and p38MAPK is required for AA-induced 5-LOX activation (Sun et al., 2019). AA is essential for the convergence of MAPK signaling cascades, which also induce the phosphorylation and activation of 5-LOX. MAPK pathways have synergistic effects on cPLA2 activation. Growth-related stimuli activate ERKs via the Raf-1/MEK1/2 protein kinase cascade and FPR2 elicits several agonist-dependent signal transduction cascades, including the Ras-MAPK pathway, PLC, and p38MAPK (Cattaneo et al., 2013a). We observed that pretreatment with SB203580, an inhibitor of the p38MAPK signaling pathway, blocked phosphorylation of 5-LOX at Ser271 (**Figure 22A**), and Ser663 phosphorylation failed when cells were preincubated with the MAPK inhibitor PD098059 (**Figure 22B**). These results indicate that FPR2 stimulation induces 5-LOX phosphorylation via FPR2-dependent p38MAPK and ERK activation. Since 5-LOX catalysis needs the oxidation of Fe^{2+} to Fe^{3+} in the active site of the enzyme, cellular redox conditions are an important modulator of 5-LOX activity. Conditions that encourage ROS generation upregulate 5-LOX, whereas reducing conditions and the presence of appropriate thiols (GSH or DTT) inhibit 5-LOX activity. ROS can also be generated by AA metabolism, released from the membrane phospholipids through cPLA2 activation. Indeed, LOX and COX-derived AA metabolites can promote ROS production by stimulating NOX, suggesting a possible signaling link between LOX/COX metabolites and NOX (Cho et al., 2011). Therefore, we investigated the role of NOX2 in FPR2-dependent phosphorylation of Ser271 and Ser663 residues of 5-LOX. We stimulated either apocynin-preincubated CaLu-6 cells and $p22phox^{Crispr/Cas9}$ cells with the FPR2 agonist and we observed that blocking of NADPH oxidase functions significantly reduces Ser271 phosphorylation (**Figure 22C and D)**. Ser663 phosphorylation was not affected by NOX-dependent modulations of redox-state (**Figure 22E and F**). Ser271 is phosphorylated by p38MAPK and we previously demonstrated that FPR2 stimulation in CaLu-6 cells induces p38MAPK activation in a NOX-dependent manner (Ammendola et al., 2021). Therefore, the enhanced release of ROS by NADPH in these cells may activate p38MAPK, which in turn mediates 5-LOX activation by increasing Ser271 phosphorylation (Werz et al., 2002). These findings show that FPR2 signaling causes 5-LOX phosphorylation at Ser271 and Ser663 residues, which require p38MAPK and ERKs activation, respectively. Furthermore, the observation that NOX2 inhibition prevents Ser271 phosphorylation demonstrates that 5-LOX translocation to the nucleus is largely dependent on FPR2-dependent NOX activation.

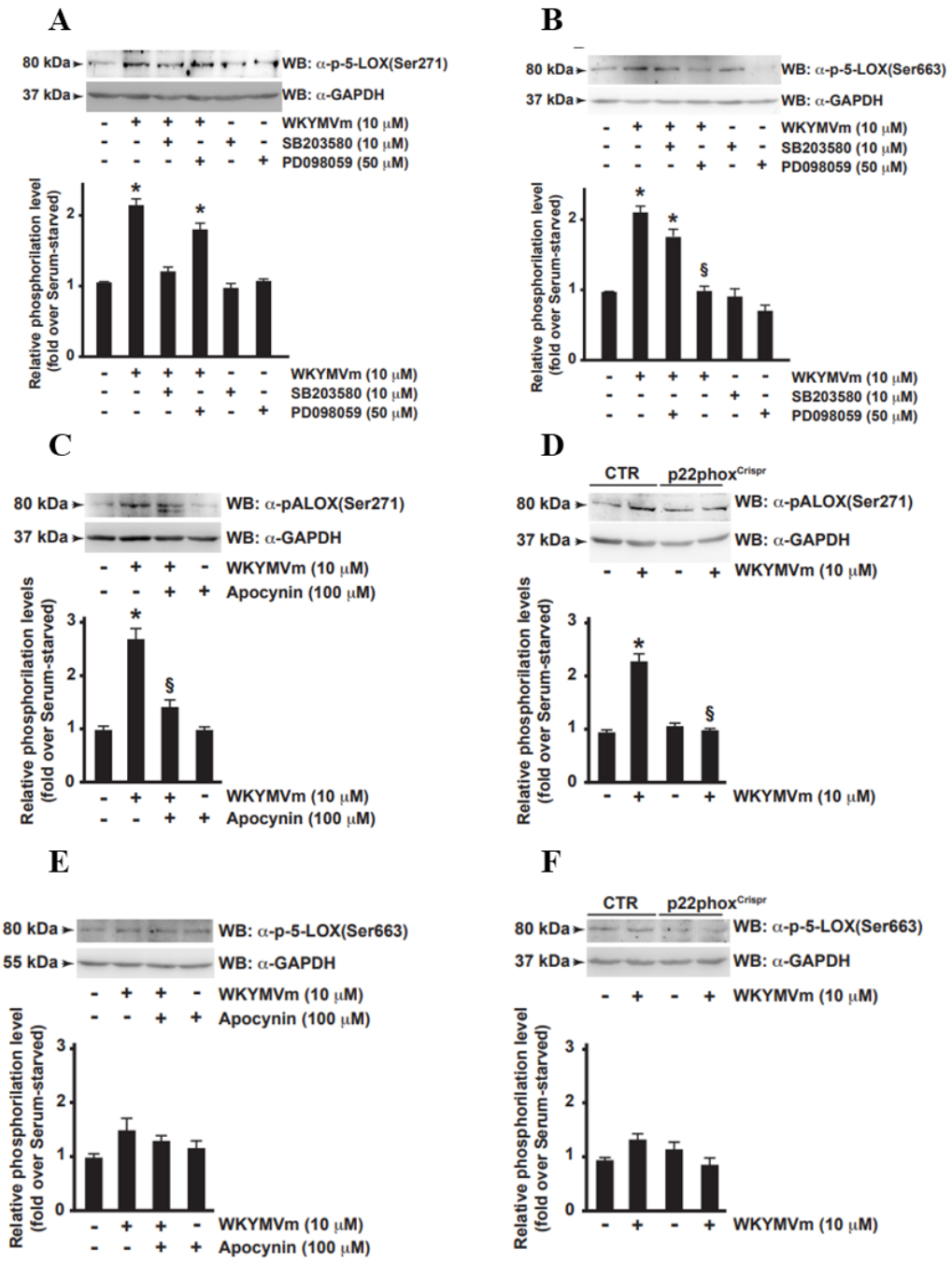

Figure 22. FPR2 signaling induces 5-LOX activation in a p38MAPK, ERKs and NOX2 dependent manner. CaLu-6 cells were serum depleted for 24h and then they are pretreated with SB203580 or PD098059 (A and B), or apocynin (C and E) before WKYMVm stimulation. p22phox$^{Crispr/Cas9}$ were serum depleted for 24h and then stimulated with FPR2 agonist (D and F). Sixty micrograms of the total lysates were loaded on 10% SDS-PAGE and then incubated with an anti-p-ALOX (Ser271) antibody (α-p-ALOX (Ser271) (A,C and D) or with an anti-p-ALOX (Ser663) antibody (α-p-ALOX (Ser663)) (B,E and F). An anti-GAPDH was utilized as a control for protein loading. Data represent three different

experiments.*$p<0.05$ in comparison to unstimulated cells. §$p<0.05$ in comparison to stimulated cells.

8. Discussion

Cancer cells have adopted a particular strategy to collect energy from the outside to sustain their rapid growth and proliferation. In fact, cancer cells, even though in presence of adequate concentration of oxygen, convert pyruvate into lactate. This effect is called *Warburg effect*. In general, tumor cells change completely their metabolism to ensure a sufficient supply of proteins, nucleotides, and lipids. Hence, metabolic reprogramming is one of the hallmarks of cancer cells. Metabolic reprogramming includes not only glucose metabolism, but also glutamine and lipids metabolism. Glucose metabolism is crucially involved in several aspects of cancer cells, since it provides intermediates and precursors for several other metabolic pathways, such as the production of amino acids, nucleotides, and lipids (Vander Heiden et al., 2009). Glutamine plays different roles in tumor cells, being used as a nitrogen donor for amino acid and nucleotide biosynthesis and for replenish TCA cycle and lipid biosynthesis pathway. Lipids represent the major components of biological membranes and the building blocks of the cell. However, they are also key signaling molecules for several cellular activities and are used as energy storage and metabolism. Therefore, tumor cells use lipids to obtain energy, membrane components, and generates signaling molecules required for proliferation, survival, inflammation, invasion and to respond to tumor microenvironment effect and cancer therapy. Furthermore, cancer cells generate a tumor microenvironment (TME), characterized by high levels of inflammation, that exacerbates malignant phenotype.

Inflammation is characterized by a high concentrations of ROS that, in turn, can act as signaling molecules and contribute to sustain inflammation. ROS are mainly produced by NOX/DUOX of NADPH oxidase family, although they are also a byproduct of mitochondria. Malignant transformation is linked to an increased ROS production and many evidences highlight the role of NOX/DUOX in cancer biogenesis (Hsieh et al., 2011).

FPR2 is a member of the Formyl peptide receptor family (FPRs). It has been initially identified in the cells of the immune system where it participates in the innate immune response. However, it is also expressed in other cell types, where it is involved in several physio-pathological conditions. For instance, FPR2 promotes the malignancy of colon cancer (Xiang et al., 2016), and induces c-Met and transactivation in PNT1A cells (Cattaneo et al., 2011) (Cattaneo et al., 2013b), respectively. In addition, FPR2 signaling triggers NOX-dependent superoxide generation in human fibroblasts IMR90 and exacerbates the malignant phenotype of CaLu-6 cells by inducing EGFR transactivation, p47phox phosphorylation, NOX-dependent superoxide generation, c-Src kinase and STAT3 activity (Cattaneo et al., 2011), suggesting a specific role of this receptor in non-phagocytic cells. Interestingly, FPR2 is associated with inflammatory

diseases since it can induce pro-inflammatory or pro-resolving response, depending on the nature of the ligands and on the specific receptor domain they recognize.

By a phoshoproteomic analysis performed in CaLu-6 cells, we observed that WKYMVm peptide, an FPR2 agonist, induced the unique phosphorylation of about 40 proteins, which are crucially involved in the energetic metabolism of CaLu-6 cells (Cattaneo et al., 2019). Therefore, in this study we analyzed the metabolic pathways activated by FPR2 agonists and the role of this receptor in metabolic reprogramming of CaLu-6 cells by using a metabolomic approach. We found that FPR2 stimulation increases concentrations of metabolites involved in glucose metabolism, glutamine metabolism, and lipid metabolism.

Our results show that FPR2 stimulation enhances glucose uptake by increasing GLUT4 expression onto the membranes of cells, in a time-dependent manner. GLUT4 is the insulin-regulated member of the GLUT family and consistently, we observed that WKYMVm stimulation triggers FPR2- and NOX2-dependent IGF-IRβ/IRβ trans-phosphorylation. The crosstalk between a GPCR and a TKR has been described in different cell lines and represents a communication system used by cells to amplify intracellular signaling cascades. Although it has been reported that IGF-IRβ can be activated by $GABA_B$, thrombin, metabotropic glutamate, neurotensin and angiotensin II type receptors, in this study we demonstrated for the first time that FPR2 functionally transactivates IGF-IR in a human cancer cell line.

We proved that FPR2 signaling directs cancer cells towards the glycolytic pathway by increasing the activity of the bifunctional enzyme PFKBP2 in a Akt- and FGFR-dependent manner. Our data demonstrate, for the first time, an important crosstalk between FPR2 and FGFR1, as well as the activation of the scaffold phosphoprotein FRS2, which acts as a docking protein downstream to FGFR1. Pyruvate deriving from glycolysis can be converted in Acetyl-CoA by pyruvate dehydrogenase (PDH) or in lactate through a reaction catalyzed by lactate dehydrogenase (LDH-A). The production of lactate and H^+ ions in cancer cells is critical for: i) the synthesis of NAD^+, which is required to sustain the increased rate of glycolysis; ii) acidification of the tumor microenvironment, which reduces the viability of normal cells and favors the infiltration of neoplastic cells (Goetze et al., 2011); and iii) binding to specific receptors on target cells, such as GPR81, which activates intracellular signaling cascades, lactate uptake, mitochondrial metabolism, tumor growth and angiogenesis (Ahmed et al., 2010) (Roland et al., 2014). Here we demonstrate that FPR2 signaling inactivates PDH by inducing its phosphorylation at Ser293 residue. Accordingly, by suppressing the oxidative decarboxylation of pyruvate FPR2 activates LDH-A, thus switching cancer metabolism towards glycolysis. LDH-A expression is modulated by c-Myc and hypoxia inducible factor-1 (HIF-1) transcriptional factors, which regulate the expression of several genes involved in glucose metabolism. HIF-1 is a heterodimer that consists of HIF-1β, which is constitutively expressed, and HIF-1α which is sensitive to oxygen concentration. In normoxic conditions HIF-1α is degraded by proteasome, whereas in

hypoxic condition it is not degraded and it translocates into the nucleus where it binds HIF-1β subunit. Once activated, HIF-1 recognizes the *hypoxia responsive element (HRE)* on target genes, including those involved in glucose uptake, glycolytic enzyme synthesis, lactate generation and secretion. NOX-dependent ROS generation is involved in hypoxic signaling in primary lung cells and, in turn, in HIF-1 stabilization (Dabral et al., 2019). Accordingly, we observed that WKYMVm peptide stabilizes HIF-1 in a time- and NOX-dependent manner. To note, FPR2 is localized not only onto the membranes of cells, but it also localizes in the nucleus of CaLu-6 and AGS cells, where it triggers c-Jun, ERKs and c-Myc activation by phosphorylation at Ser62 residue. Our results show a significant increase of c-Myc Ser 62 phosphorylation in a time- and FPR2-dependent manner, proving that FPR2 regulates glucose metabolism by controlling HIF-1 and c-Myc activation.

In our metabolomic analysis of FPR2-stimulated CaLu-6 cells we also observed an increased concentration of ribose-5P, citrate, malate, aspartate, glutamate, glutamine, and pyrimidinic nucleotides. Here, we demonstrate that FPR2 signaling activates CAD, the multifunctional enzyme that catalyzes the three initial speed limiting steps of the *de novo* synthesis of pyrimidinic nucleotides. In particular, FPR2 signaling triggers CAD phosphorylation at Ser1859 residue, assisting cells progression through the S phase of the cell cycle. Blockade of NOX2 functions prevents CAD phosphorylation, proving that it requires NOX2-dependent ROS generation.

In FPR2-stimulated CaLu-6 cells we also observed an enhanced concentration of ribose 5P, which indicates the entrance of glucose-6P in the PPP. In fact, this pathway uses glucose-6P as a primary substrate and it consists of an oxidative phase and a non-oxidative phase. The first one is characterized by three irreversible reactions that produce ribose-5P and NADPH, whereas the non-oxidative phase consists of several reversible reactions catalyzed by TKT and TALDO that recruit glycolytic intermediates that can be converted in pentose phosphates and vice versa. Accordingly, to the observed enhanced concentration of ribose-5P, we prove that FPR2 signaling induces a time-dependent increase of NAPDH, thus suggesting that FPR2 triggers the oxidative phase of PPP. The increased concentrations of NAPDH and ribose-5P are required by tumor cells for reductive biosynthesis of macromolecules and ribonucleotides synthesis, respectively. Since reactions of non-oxidative phase are reversible, they can feed the production of ribulose-5P and, in turn, of ribose-5P. Accordingly, we observed an increase of TKT activity in FPR2-stimulated CaLu-6 cells.

Glutamine is the most prevalent amino acid, and it provides cancer cells carbon and nitrogen to sustain biosynthesis, metabolic activities, and cellular homeostasis. ASCT2, a glutamine transporter, is overexpressed in several types of cancer and correlates with increased glutamine uptake. We demonstrate that FPR2 signaling increases ASCT2 expression in a time-dependent manner via c-Myc activation. Glutamine is also important in mitochondrial metabolism, where a particular variant of glutamine transporter, the SLC1A5 gene, has been identified. Upon its entry into the cell,

glutamine is converted into glutamate by glutaminase, which can be transformed into αKG by aminotransferases or glutamate dehydrogenase. αKG can feed TCA cycle thus increasing ATP and citrate concentration. Accordingly, in FPR2-stimulated cells, we observed an increase of citrate concentration and changes in mitochondrial membrane potential. The enhanced concentration of malate, noted in FPR2-stimulated cells, suggests a direct connession with the observed increase of NADPH. In fact, malate is the substrate of the malic enzyme to produce pyruvate and NADPH. Furthermore, in cells that undergo continuous mitosis, the observed increase of aspartate is necessary for nucleotides synthesis. Therefore, oxaloacetate can exit by TCA and converted to aspartate by transaminases.

Besides glucose and glutamine metabolism, in recent years it has been reported that also lipid metabolism is involved in metabolic reprogramming of cancer cells. Accordingly, we observed a significant increase of arachidonic acid (AA) concentration in FPR2-stimulated cells. cPLA2 enzymatic cleavage of the sn-2 position of exiting membrane phospholipids represent one of the main mechanisms utilized by cells to obtain AA. cPLA2 is regulated by an increased concentration of intracellular Ca^{2+} and lipids which activate the enzyme and enhance its residence time in membranes. Furthermore, in cPLA2 three phosphorylation sites regulate the enzyme activity. In particular. Ser505 residue is responsible of its activity and its expression in the membrane. We show that WKYMVm stimulation enhances cPLA2 phosphorylation at Ser505 residue in a FPR2- and NOX2-dependent manner. AA can be metabolized by lipoxygenases (LOXs) that produce mainly leukotrienes (LTs), by cytochrome p450 and by cyclooxygenases (COXs) that produce prostaglandins (PGs). LOXs comprises three different isoforms: 12-lipoxygenase, 15-lipoxygenase and 5-lipoxygenase. Among these isoforms, 5-LOX has been reported hyper-expressed in several types of cancers (Hennig et al., 2005) (Wang & Dubois, 2010) where it is also involved in cell differentiation, oxidative stress and in the progression of different diseases (Wang et al., 2017) (Mashima & Okuyama, 2015) (Chen et al., 2023). In resting cells, 5-LOX localizes into the cytosol, but upon an activating stimulus it translocates into the nucleus. 5-LOX is a substrate of different protein kinases, and its phosphorylation at different residues has distinct effects on 5-LOX subcellular distribution and activity. Phosphorylation at Ser271 and Ser663 residues enhance 5-LOX synthesis and are required for 5-LOX activation. We prove that WKYMVm stimulation enhances 5-LOX phosphorylation on these residues in a time-dependent manner. Ser271 is a phosphorylation target of p38MAPK, whereas Ser663 is phosphorylated by ERKs. Activated FPR2 triggers multiple signaling cascades, including Ras-MAPK pathway, PLC, and p38MAPK (Cattaneo et al., 2013a). Accordingly, our results show that preincubation with a p38MAPK or a MAPK inhibitor, prevent phosphorylation of 5-LOX at Ser271 or Ser663 residues. Since 5-LOX activation requires the oxidation of Fe^{2+} to Fe^{3+}, cellular redox conditions are involved in 5-LOX activation. Therefore, conditions that enhance ROS concentration upregulate 5-LOX, whereas reducing conditions and the presence of appropriate thiols inhibit 5-LOX activity. ROS can also be generated by AA metabolism and released from

membrane phospholipids via cPLA2 activation. Indeed, LOX and COX-derived AA metabolites can stimulate NOX and promote ROS generation, showing a putative signaling relationship between LOX/COX metabolites and NOX (Cho et al., 2011). Accordingly, our results prove that Ser271 phosphorylation is significant reduced after NOX2 blockade,

This metabolomic approach is the first study that evaluates the role of FPR2 in metabolic reprogramming of CaLu-6 cells and prove the contribute of this receptor in modulating glucose, glutamine, arachidonate and nucleotide metabolism. Therefore, FPR2 could be considered as a new potential therapeutic target able to modulate cancer cells metabolism.

9. Conclusions

Growing evidences suggest that complex signal transduction networks participate to reconfiguration of metabolic processes of cancer cells. However, the integration of such signaling pathways and cellular metabolism remains to be clarified. This study sheds the light on new molecular mechanisms through which FPR2/TKR crosstalk signaling and NOX2-dependent ROS production regulate glucose, glutamine and lipid metabolism in CaLu-6 cells. FPR2 stimulation triggers intracellular signaling cascades that result in TKR transactivation, insulin-dependent glucose uptake, activation of regulatory glycolytic enzymes, promotion of aerobic glycolysis for energy production, and increased lactate production. FPR2 also enhances glutamine uptake and promotes PPP and synthesis of pyrimidine nucleotides. FPR2 signaling plays a key role in arachidonic acid cascade in cancer cells, thus contributing to inflammatory processes and upregulating cancer cell proliferation, survival, migration and promotion of tumor development. This study shows, for the first time, the contribution of FPR2 to the cellular metabolic activities of proliferating cells and point out the crucial role of FPR2/NOX-mediated redox signaling on the regulation of several of these processes, suggesting a promising role of this receptor as therapeutics targets.

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11. List of publications

- Caso, V. M., Manzo, V., Pecchillo Cimmino, T., Conti, V., Caso, P., Esposito, G., Russo, V., Filippelli, A., Ammendola, R., & Cattaneo, F. (2021). Regulation of Inflammation and Oxidative Stress by Formyl Peptide Receptors in Cardiovascular Disease Progression. *Life (Basel, Switzerland)*, *11*(3), 243. https://doi.org/10.3390/life11030243
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