ROS mediates interferon gamma induced phosphorylation of Src, through the Raf/ERK pathway, in MCF-7 human breast cancer cell line

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Abstract Interferon gamma (IFN- γ) is a pleiotropic cytokine which plays dual contrasting roles in cancer. Although IFN- γ has been clinically used to treat various malignancies, it was recently shown to have protumorigenic activities. Reactive oxygen species (ROS) are overproduced in cancer cells, mainly due to NADPH oxidase activity, which results into several changes in signaling pathways. In this study, we examined IFN- γ effect on the phosphorylation levels of key signaling proteins, through ROS production, in the human breast cancer cell line MCF-7. After treatment by IFN- γ , results showed a significant increase in the phosphorylation of STAT1, Src, raf, AKT, ERK1/2 and p38 signaling molecules, in a time specific manner. Src and Raf were found to be involved in early stages of IFN- γ signaling since their phosphorylation increased very rapidly. Selective inhibition of Src-family kinases resulted in an immediate significant decrease in the phosphorylation status of Raf and ERK1/2, but not p38 and AKT. On the other hand, IFN- γ resulted in ROS generation, through H₂O₂ production, whereas pre-treatment with the ROS inhibitor NAC caused ROS inhibition and a significant decrease in the phosphorylation levels of AKT, ERK1/2, p38 and STAT1. Moreover, pretreatment with a selective NOX1 inhibitor resulted in a significant decrease of AKT phosphorylation.

Finally, no direct relationship was found between ROS production and calcium mobilization. In summary, IFN- γ signaling in MCF-7 cell line is ROS-dependent and follows the Src/Raf/ERK pathway whereas its signaling through the AKT pathway is highly dependent on NOX1.

Keywords IFN- γ · MCF-7 · STAT1 · Src · Raf · p38 · ERK1/2 · AKT · ROS · NOX · Cancer · Signal transduction

Introduction

Interferon gamma (IFN- γ) is a pleiotropic cytokine whose biological activity mediates cytostatic/cytotoxic effects and anti-tumor mechanisms. It plays an important role in intercellular communication during innate and acquired immune responses in addition to host defense against viral and bacterial infections (Min et al. 1996). Although IFN- γ has been clinically used to treat a variety of malignancies, it was recently shown to have protumorigenic activities (Lollini et al. 1993). This contrasting behavior of IFN- γ depends on the cellular, molecular and microenvironment context. IFN- γ is the only member of type 2 interferon and is restrictively expressed. IFN- γ activates several different kinases and transcription factors including phosphorylation of signal transducer and activator of transcription 1 (STAT1) through non-receptor tyrosine kinases, Janus activated kinase (JAK) 1 and 2, promoting STAT1 dimerization and its translocation into the nucleus. This leads to the activation of several genes (Zhong et al. 2005) such as NADPH oxidase (NOX) (Sesti et al. 2012; Droge 2002) and cell cycle regulatory proteins (Subramaniam and Johnson 1997; Resnitzky et al. 1992). Indeed, the antiparallel form of IFN- γ dimer interacts with the extracellular domain of the IFN- γ R1 receptor subunit that will engage

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to the IFN- γ R2 receptor subunit (Ealick et al. 1991), inducing the canonical IFN- γ pathway, known as IFN- γ /JAK/STAT (Platanias 2005).

Moreover, proinflammatory factors such as tumor necrosis factor alpha (TNF- α) and granulocyte-macrophage colony stimulating factor (GM-CSF), microbial products such as lipopolysaccharides (LPS) (Cassatella 1990), and cytokines such as IFN- γ , stimulate several reactive oxygen species (ROS) producing enzymes leading to inflammatory oxidative stress (Cadwallader 2002). In fact, cytokines, including IFN- γ , induce ROS production through NADPH oxidase (NOX) (Lambeth 2004) which may lead to several modifications of intracellular signaling pathways, by affecting the activities of kinases and phosphatases (Trachootham et al. 2008; Sundaresan et al. 1995). ROS generation is mostly derived from the metabolism of oxygen (Halliwell 1999), whose harmful effects result from its metabolic reduction to highly reactive and toxic molecules (Buechter 1988). There are numerous potential sources of ROS within the cell including NOX enzymes that produce oxidants as part of their normal enzymatic function. For instance, neutrophils and macrophages catalyze the generation of ROS by NOX, a mechanism known as respiratory burst (Babior et al. 2002). Cancer cells are also characterized by overproduction of ROS due to the activity of several ROS producing enzymes including mitochondrial electron transport chain, cytochrome P450 and most importantly NADPH oxidase (NOX).

ROS were historically viewed as purely harmful; however, they are currently observed as important physiological regulators of intracellular signaling pathways. In fact, ROS can modulate the activities and expression of many transcription factors and signaling proteins that are involved in cell survival and stress response (Trachootham et al. 2008). On the other hand, overproduction of ROS and changes in intracellular antioxidant levels can lead to pathological and cytotoxic effects (Sesti et al. 2012). Therefore, maintaining ROS homeostasis, through enzymatic and non-enzymatic antioxidant systems, is crucial for normal cell growth and survival (Droge 2002). Indeed, a disruption of redox homeostasis is associated with abnormal cancer cell growth due to either an elevation of ROS production or to a decline of their scavenging capacity, a condition known as oxidative stress (Toyokuni et al. 1995). In order to counteract increased ROS generation and to maintain redox homeostasis, cancer cells mediate redox adaptation by activating ROS scavenging enzymes (Pervaiz 2004).

In this study, we examined the effect of IFN- γ on the phosphorylation levels of key signaling proteins such as STAT1, Src, Raf, p38 and ERK1/2 MAP-Kinases, and AKT, through ROS production and calcium mobilization.

Materials and methods

Cell lines and culture conditions Human breast cancer cell line (MCF-7) was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies) and 1% Penicillin-Streptomycin (PS; Sigma-Aldrich, USA) and incubated at 37 °C in 5% CO₂. Cell cultures were used within 10 passages from recovery to obtain reproducible and representative results.

Reagents IFN- γ (Sigma) was used at 5nM, unless otherwise specified. All inhibitors were used at 10 μ M concentrations and were purchased from Sigma (USA). These included the ROS inhibitor NAC (N-acetyl cysteine), the kinase inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine), the NOX1 inhibitor ML171 (2-acetylphenothiazine), NOX2 inhibitor, and the NOX inhibitor VAS2870 (3-Benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5-d)pyrimidine).

RNA extraction Total RNAs from cultured cells were isolated using TriPure kit according to manufacturer's instructions (Roche, USA). The quantity of RNA was measured using the NanoDrop® ND-1000 UV-vis Spectrophotometer. RNA purity was assessed using the absorbance ratio of 260 to 280 nm, where a value of 1.8–2.0 indicated good quality RNA. Finally, RNAs were treated by Deoxyribonuclease I (Fermentas, USA) according to manufacturer's instructions followed by RNA precipitation and quantification.

RT-PCR Reverse transcription was carried out on 1 μ g of RNA, using the qScript cDNA™ SuperMix (Quanta Biosciences, USA), according to manufacturer's instructions. The resulting cDNA was then used for PCR using the following primers: IFN- γ R, F: CATCACGTCATACCAGCCATTT and R: CTGGATTGTCTTCGGTATGCAT; GAPDH, F: 5'-GTGTTCCTACCCCCAATGTGT-3', R: 5'-ATTGTCATACCAGGAAATGAGC-3'. The PCR conditions were 95 °C for 3 min, then 40 cycles each of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min. Results were analyzed by agarose gel electrophoresis. Negative control (water without DNA) was used to check for contamination.

Protein extraction and quantification Proteins from cultured cells were homogenized with RIPA buffer (1 M Tris, 4 M NaCl, 0.5 M EDTA, 1% NP40, and 0.1% sodium dodecyl sulfate [SDS]) containing protease and phosphatase inhibitors (Roche Applied Science, Germany), in the presence or absence of different concentrations of NAC (1 mM or 10nM). The lysates were incubated for 20 min on ice followed by

centrifugation at 11,000 rpm for 15 min at 4 °C and protein concentrations determined using Bradford Assay.

Western blotting Protein samples (50 μ g each) were mixed with loading buffer (Lamaelli and β -Mercapto-ethanol), heated at 95 °C for 10 min and then loaded into the wells of stacking gel for electrophoresis. Gels were then transferred to PVDF membranes (Bio-Rad, Germany) at 4 °C at 80–100 volts for 1 h. The membrane was then blocked in 5% fat-free milk, prepared in wash buffer (1 M Tris-HCl, pH 8.8, 5 M NaCl, and 0.05% Tween 20), overnight at 4 °C. Detection of the protein of interest was achieved by probing the membrane with the primary antibody of interest. Monoclonal antibodies used were for mouse anti- p-STAT1, p-p38, p-ERK1/2, p-AKT, p-Src, p-Raf (Santa Cruz, Germany; 1:1000 dilution). GAPDH antibody were used to ensure equal loading of samples (1:3,000 dilution). After several washes, the membrane was then incubated in horseradish peroxidase-conjugated anti-rabbit secondary antibody (diluted at 1:10,000) for 1 h at RT. Protein bands were visualized using a chemiluminescent detection system (FluorChem E FE0324, ECL). Molecular weight markers enabled the determination of protein sizes. Intensity of bands was then determined by densitometry, using ImageJ software. Band Intensities for each signaling molecule was then normalized against GAPDH levels.

NOX experiment In order to study the implication of NOX, MCF-7 cells were cultured at 10^6 cells/well and pretreated with various NOX inhibitors at 10 μ M for 1 h, before treatment with 5nM IFN- γ for 5 min and assessment of the phosphorylation levels of AKT by western-blot, as indicated above. The following specific inhibitors were used: NOX1 inhibitor ML171, NOX2 inhibitor, and NOX3 inhibitor VAS2870.

Assessment of H₂O₂ production MCF-7 cells were incubated at 37 °C for 20 min with 20 mM 2,7-dichlorofluorescein (DCFH) diacetate (Molecular Probes, USA). After labeling, cells were treated, and production of H₂O₂ was then assessed every 10 min by measuring DCF emission at 525 nm; using a fluorometer (LS50B fluorescence spectrometer, Perkin Elmer, USA). Results were expressed as relative mean fluorescence intensity $\pm$ SEM.

Calcium measurement MCF-7 cells were loaded with the calcium-sensitive dye Fluo-3/AM and free intracellular calcium was determined with a LS50B fluorescence Photometer (Perkin Elmer, USA). Calcium concentration was calculated using the equation: $[Ca^{2+}] = \{(dF - F_{min}) / (F_{max} - dF)\} * K_d$ where dF is the observed fluorescence, F_{min} is the fluorescence at low Ca²⁺ (5 mM EGTA), F_{max} is the fluorescence at high Ca²⁺ (8 μ M ionomycin), and K_d being 390 nM for Fluo-3/AM. Values for F_{min} and F_{max} were obtained at the end of each

experiment. Peak values were recorded and plateau phases calculated as the area under the curve, starting at the peak and up to 200 s thereafter, and expressed as arbitrary units.

Statistical analysis Results are expressed as individual data or as the mean $\pm$ SEM. Statistical comparisons were performed using one way ANOVA in order to determine statistical significance. Student's *t*-test gave similar results to ANOVA. The *p* value was determined and values for *p* < 0.05, *p* < 0.001, and *p* < 0.0001 were considered significant (*, **, ***; respectively). Microsoft Excel and GraphPad software were used to perform statistical analysis.

Results

IFN- γ -induced phosphorylation of STAT1, Src, Raf, MAP Kinases and AKT In order to evaluate whether IFN- γ could induce any signaling pathway in MCF-7 cell line, the expression of interferon gamma receptor type 1 (IFN- γ R₁) was first explored within these cells at the transcriptional and translational levels. Indeed, IFN- γ R1 was shown to be expressed in MCF-7 cells at the mRNA and protein levels, by RT-PCR and

Fig. 1 Expression of IFN- γ R₁. The MCF-7 cell line was investigated for the expression of IFN γ R₁ mRNA and proteins using: (a) RT-PCR. (b) Western blot and (c) Flow cytometry analysis. The green peak represents the shift resulting from labeling with an antibody against IFN γ R1 in MCF-7 cells. The other peak corresponds to a negative control cell line (RAW cells) which does not express IFN γ R1. The RAW cell line was used as a negative control whereas the THP-1 cells were used as a positive control

Western blot, respectively (Fig. 1a and b). Furthermore, its protein expression was confirmed by flow cytometry after treatment with 5nM IFN- γ (Fig. 1c).

To investigate the signalling pathways induced by IFN- γ , we examined the variation of phosphorylation status for STAT1, a main transactivator in the IFN- γ signaling pathway (Platanias 2005), Src, Raf, AKT, ERK1/2 and p38. Results showed that upon treatment of MCF-7 cells with 5nM IFN- γ , STAT1 was immediately phosphorylated and reached a peak of expression at 10 min (~12 fold increase, **, $p < 0.001$), which decreased gradually to its almost original levels after 2 h (Fig. 2a).

In addition, the phosphorylation status of Src and Raf proteins were then explored in order to confirm their involvement as immediate positive regulators of IFN- γ signaling pathway. In fact, upon treatment with 5nM IFN- γ , Src was phosphorylated within seconds, reached a peak of expression at 1 min (~10 fold increase, ***, $p < 0.0001$), and decreased gradually and significantly at 30 min (Fig. 2b). Moreover, there was an increase in the phosphorylation levels of Raf, which reached a peak after 10 min of treatment with IFN- γ (~10 fold increase, ***, $p < 0.0001$), and was maintained for 1 h (Fig. 2c).

Furthermore, AKT phosphorylation was maximally observed after 5 min of treatment with IFN- γ (~10 fold increase,

***, $p < 0.0001$) which decreased rapidly to reach its original levels at 2 h (Fig. 2d). Finally, there was a similar, but slower, gradual increase in the phosphorylation levels of MAPK members ERK1/2 (~10 fold increase, **, $p < 0.001$) and p38 (~13 fold increase, ***, $p < 0.0001$) which reached a peak after 20 min of treatment with IFN- γ (Fig. 2e and f, respectively). This maximal phosphorylation of ERK1/2 was maintained for 1 h whereas that of p38 for 30 min and decreased slowly thereafter, confirming that ERK1/2 and p38 proteins are major players in IFN- γ signaling pathway (Fig. 2e and f, respectively). It's important to note that p-Src was the only signaling molecule that appeared very early (within seconds) whereas STAT1, Raf, AKT, ERK1/2 and p38 started to appear at a later time (data not shown).

These results indicate that the increased phosphorylation of STAT1, Raf, Src and AKT in response to IFN- γ signaling in MCF-7 cells might play important roles at early stages of IFN- γ signalling. This suggests that Src and Raf activation in MCF-7 cells is upstream of other INF- γ induced signaling proteins.

Src mediates IFN- γ -induced phosphorylation of Raf and ERK, but not p38 and AKT Pretreatment of MCF-7 cells for 1 h with 10 μ M PP2, a selective inhibitor of Src-family kinases, followed by treatment with 5nM IFN- γ resulted in an

Fig. 2 IFN- γ induces the phosphorylation of STAT1, Src, Raf, AKT, ERK1/2 and p38. Western blot analysis showing the effect of IFN- γ on the phosphorylation levels of different signaling proteins. A total of 10^6 MCF-7 cells/well were cultured in the presence of 5nM IFN- γ at different time intervals. Protein expression levels and histogram analysis showing the relative levels of expression at different times for: (A) p-STAT1 (B) p-Src (C) p-Raf (D) p-AKT (E) p-ERK1/2 (F) p-p38. Quantification was

done by measuring the intensity of each band by densitometry, using ImageJ software. Values represent the average fold change, normalized to GAPDH, and relative to control. Results are representatives of three independent experiments ($n = 3$), for each time point and treatment condition, reported as the mean plus or minus the standard error of the mean (mean $\pm$ SEM). *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

immediate significant decrease (~ 5 fold; **, $p < 0.001$.) in the phosphorylation status of both Raf and ERK proteins (Fig. 3a and b). On the other hand, the IFN- γ induced p-p38 and p-AKT activation was not inhibited by the addition of PP2 (Fig. 3c and d, respectively). These results confirmed that IFN- γ signaling is src-dependent and follows the Src/Raf/ERK pathway, rather than the PI3K/AKT pathway in MCF-7 cells.

IFN- γ induced ROS through H₂O₂ production We then examined the effect of IFN- γ on H₂O₂ production. In order to achieve this goal, different doses of IFN- γ treatment were first used to establish the optimal IFN- γ concentration for H₂O₂ production in MCF-7 cells. Concentrations of 50 nM and 5 nM gave similar significant responses (~ 3 –4 fold increase, **, $p < 0.001$) (Fig. 4a and b). The amount of H₂O₂ produced after 5nM IFN- γ treatment was then measured in the presence or absence of N-acetyl cysteine (NAC), a ROS inhibitor. MCF-7 cells showed a significant ~ 3 fold increase in H₂O₂ production following treatment with 5nM IFN- γ , in comparison to basal

control levels (**, $p < 0.001$, Fig. 4c and d). On the other hand, pretreatment with 1 mM NAC, but not 10 μ M, showed a significant decrease in H₂O₂ production, compared to cells treated with IFN- γ alone. Our results confirmed that IFN- γ signaling pathway induces ROS, through H₂O₂ production, which was inhibited by 1 mM NAC.

ROS mediates IFN- γ -induced phosphorylation of AKT, ERK, p38 and STAT1 Since ROS production is induced by IFN- γ , we then investigated the consequences of specifically inhibiting IFN- γ -induced ROS production on INF- γ induced activation of different signaling pathways, by pretreating MCF-7 cells in the presence or absence of 1 mM, or 100 μ M, NAC for 1 h. As determined above, cells were treated with 5nM IFN- γ for the optimal time of 5, 20, 20, or 10 min, respectively, in order to reach a peak of expression for AKT, ERK, p38, and STAT1.

Our results confirmed the previously observed IFN- γ induced significant increase in the phosphorylation levels of these signaling proteins, in comparison to controls (Fig. 5).

Fig. 3 PP2 inhibits the phosphorylation of Raf and ERK, but not p38 and Akt. Western blot analysis showing the effect of PP2, the selective inhibitor of Src-family kinases, on the phosphorylation levels of different signaling proteins. MCF-7 cells were cultured as 10^6 cells/well and pretreated with 10 μ M PP2 before treatment with 5nM of IFN- γ . Protein expression levels and histogram analysis showing the relative levels of expression at different times for: (a) p-Raf (b) p-ERK (c) p-

p38 (d) p-AKT. Quantification was done by measuring the intensity of each band by densitometry, using ImageJ software. Values represent the average fold change, normalized to GAPDH, and relative to control. Results are representatives of three independent experiments ($n = 3$), for each time point and treatment condition, reported as the mean $\pm$ SEM. *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

Fig. 4 Measurement of H₂O₂ production using fluorometry after IFN- γ treatment in the presence or absence of NAC. MCF-7 cells were cultured as 10^6 cells/well and pretreated with 1 mM or 10 nM of NAC before treatment with 5 nM of IFN- γ . Following that, H₂O₂ production was measured. **a** Time course for the relative fluorescence of H₂O₂ production measurement for MCF-7 cells treated with 1 nM, 5 nM or 50 nM IFN- γ for 30 min. **b** Quantification of the relative fluorescence

curves at 30 min. **c** Time course for the relative fluorescence of H₂O₂ production measurement for MCF-7 cells treated with 5 nM IFN- γ , with or without NAC. **d** Quantification of the relative fluorescence, with or without NAC. Results are representatives of three independent experiments ($n=3$) reported as the mean $\pm$ SEM. *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

However, pretreatment of MCF-7 cells with 1 mM NAC, but also 100 μ M NAC, caused AKT phosphorylation to decrease significantly, compared to cells treated with 5 nM IFN- γ alone (**, $p < 0.001$, Fig. 5a). Moreover, similar results were obtained for ERK1/2 and p38 MAP kinases, but also for STAT1 phosphorylation, which were significantly inhibited after pretreatment with 1 mM NAC, but to a lesser extent with 100 μ M, in comparison to cells treated with IFN- γ alone (**, $p < 0.001$, Fig. 5b, c and d, respectively). These results confirmed that ROS is an important positive regulator of AKT, ERK1/2, p38 and STAT1 phosphorylation and also an activator of IFN- γ signaling pathway.

INF- γ induced-phosphorylation of AKT is highly dependent on NOX-1 activity To investigate the role of AKT on NADPH oxidase (NOX) activation, phosphorylated AKT were investigated in the presence of different specific NOX inhibitors. Indeed, pretreatment of cells with ML171, a selective inhibitor of NOX1, followed by treatment with 5 nM IFN- γ resulted in a significant decrease (~ 4 fold) in the phosphorylation status of AKT (*, $p < 0.05$, Fig. 6a). On the other hand, the increase in p-AKT, following IFN- γ treatment, was not

inhibited by the addition of specific NOX2 inhibitor or the new NOX inhibitor VAS2870 (Fig. 6a). These results confirm that IFN- γ signaling through the AKT pathway is highly dependent on Nox1. In order to further confirm our data, ROS was directly modified using H₂O₂ which caused a gradual activation of Akt, by phosphorylating it (Fig. 6b).

INF- γ induced-intracellular calcium rise is not affected by NAC In order to establish the optimal concentration for the measurement of intracellular calcium, MCF-7 cells were first labelled with Fluo-3/AM and treated with different doses of INF- γ . Concentrations of 5 and 50 nM INF- γ gave similar significant responses in the rise of calcium levels (***, $p < 0.0001$, Fig. 7a and b). Therefore, MCF-7 cells were then treated with 5 nM INF- γ to study the effect of ROS production on intracellular calcium mobilization. Data showed a significant rise in calcium levels which peaked at ~ 300 nM (Fig. 7c and d). However, pretreatment with 1 mM NAC showed a similar effect to that observed by INF- γ treatment in the absence of NAC. This indicates that ROS didn't have a major role in intracellular calcium rise, since

Fig. 5 IFN- γ induced phosphorylation of AKT, ERK1/2, p38, and STAT1 is inhibited in the presence of NAC. Western blot analysis showing the effect of IFN- γ on the phosphorylation levels of different signaling proteins, in the presence of NAC. MCF-7 cells were cultured as 10^6 cells/well and pretreated with 1 mM or 100 μ M NAC before treatment with 5nM of IFN- γ . Expression levels and histogram analysis showing the relative levels of expression at different times for: (A) p-AKT (B) p-ERK (C) p-p38 (D) p-STAT1. It's worth noting that LPS

produced a similar increase in phosphorylation of the above signaling molecules to that observed after IFN- γ treatment and hence was used as a positive control. Quantification was done by measuring the intensity of each band by densitometry, using ImageJ software. Values represent the average fold change, normalized to GAPDH, and relative to control. Results are representatives of three independent experiments ($n = 3$), for each time point and treatment condition, reported as the mean $\pm$ SEM. *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

inhibition of ROS by 1 mM of NAC didn't cause any significant change in calcium levels.

Discussion

IFN- γ is a cytokine that plays important roles in intercellular communication during innate and adaptive immune responses as well as antitumor effect (Platanias 2005). IFN- γ molecular signalling are mediated by the sequential phosphorylation of different molecules including STATs and JAKs (3, 20). In this study, the signaling effects of IFN- γ in MCF-7 cells were evaluated by investigating the phosphorylation/activation status of

STAT1, Src, Raf, AKT, ERK1/2 and p38 MAP kinases, in addition to ROS production.

This study demonstrated two major novel findings (Fig. 8). First, IFN- γ induced the phosphorylation of different signaling molecules in MCF-7 cells including STAT1, Src, Raf, AKT, ERK1/2, and p38 in a time specific manner, under the effect of ROS activity. Second, IFN- γ signaling follows the Src/Raf/ERK pathway and is ROS-dependent whereas ROS implication through the AKT pathway is highly dependent on NOX1 activity. The following several lines of evidence support the above statements: (1) IFN- γ R₁ was confirmed to be transcriptionally expressed in MCF-7 cell

Fig. 6 ML171, a selective NOX1 blocker, inhibits the phosphorylation of AKT. **a** Western blot analysis showing the effect of various NOX inhibitors on the phosphorylation levels of AKT. MCF-7 cells were cultured as 10^6 cells/well and pretreated, or not, with $10 \mu\text{M}$ of specific inhibitors (ML171, NOX2 inhibitor, and VAS2870) before treatment with 5nM of IFN- γ . Protein expression levels and histogram analysis showing the relative levels of expression at 10 min for p-AKT. **b** Time course of

Akt phosphorylation when ROS was directly modified using $10 \mu\text{M}$ H_2O_2 . Results showed that H_2O_2 gradually activated Akt. Values represent the average fold change, normalized to GAPDH, and relative to control. Results are representatives of three independent experiments ($n = 3$), for each time point and treatment condition, reported as the mean $\pm$ SEM. *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

line. (2) IFN- γ treatment induced the phosphorylation of STAT1, p38, ERK1/2 and AKT which appeared within minutes, reached a maximum at 10-20 min, and started to decrease thereafter. (3) IFN- γ significantly increases the phosphorylation levels of Src, within seconds, and Raf, within minutes, which are involved in early stages of IFN- γ signaling pathway. (4) Selective inhibition of Src-family kinases resulted in an immediate significant decrease in the phosphorylation status of both Raf and ERK, but not p38 and AKT. Therefore, IFN- γ signaling is Src-dependent and follows the Src/Raf/ERK pathway. (5) IFN- γ significantly induced ROS, through H_2O_2 production, which was significantly inhibited by pretreatment with the ROS scavenger NAC. (6) Pretreatment with NAC significantly decreased the phosphorylation of AKT, ERK1/2, p38, and STAT1. (8) Pretreatment with selective inhibitors of NOX1 resulted in a significant decrease in the phosphorylation status of AKT. Therefore, ROS implication in IFN- γ signaling through the AKT pathway is highly dependent on NOX1.

STATs were originally described as growth factors and are involved in the regulated expression of many proteins underlying diverse cellular signalling including growth and apoptosis. Several studies have shown that STAT1 is phosphorylated in response to interferons (Platanias 2005) and it is immediately activated by

non-receptor tyrosine kinases known as Janus activated kinase (JAK) 1 and 2 (Zhong et al. 2005; Haan et al. 2006). In addition to the rapid activation of STAT1 in response to IFN- γ signaling, STAT1 is also activated by other kinases, including PI3K/AKT and MAP kinases such as ERK1/2 and p38. Moreover, PI3K/AKT and MAP kinases were also shown to be important positive regulators of IFN- γ signaling pathway since they could also phosphorylate STAT1 on serine residues, in order to enhance its translocation into the nucleus (Krämer et al. 2009). Our data are in accordance with previous studies on solid tumors which showed that IFN- γ induces phosphorylation of tyrosine STAT1, which forms STAT1 homodimers through the activity of Janus activated kinase (Zhong et al. 2005). The slight decrease in p-STAT1 after 2 h may be due to a negative feedback loop, in order to downregulate IFN- γ signaling pathway. It was shown that IFN- γ reversed its effect on p38 phosphorylation. This effect of IFN- γ could be due to an activation of another signaling pathway that may activate MAP Kinases Phosphatases (MKP). The latter might dephosphorylate MAP Kinases, including p38.

In epithelial cells, IFN- γ promotes the activation of NOX enzymes (Cassatella 1990; Kuwano et al. 2006) which will then produce superoxide anion that is further converted to H_2O_2 (Finkel 2011). H_2O_2 is another form of ROS which

Fig. 7 Measurement of intracellular calcium after treatment with IFN- γ in the presence or absence of NAC. MCF-7 cells were cultured as 10^6 cells/well and treated with 5nM of IFN- γ , in the presence or absence of 1 mM NAC. Fluorometry was then used in order to measure intracellular calcium. **a** Time course for the relative fluorescence of calcium measurement for MCF-7 cells treated with 0.1nM, 1nM, 5nM or 50nM IFN- γ for 30 min. **b** Quantification of the relative fluorescence curves at

30 min. **c** Time course for the relative fluorescence of calcium measurement for MCF-7 cells treated with 5nM IFN- γ , with or without NAC. **d** Quantification of the relative fluorescence, with or without NAC. Results are representatives of three independent experiments ($n=3$), for each time point and treatment condition, reported as the mean reported as the mean $\pm$ SEM. *, **, *** indicate $p < 0.05$, $p < 0.001$, $p < 0.0001$; respectively

are carcinogens and have been detected in almost all cancers (Sundaresan et al. 1995; Toyokuni et al. 1995). In addition, recent studies revealed that several cytokines including IFN- γ could induce ROS production through the activity of a membrane bound enzyme known as NADPH oxidase (NOX) (Cassatella 1990; Lambeth 2004). Moreover, ROS was shown to be involved in several intracellular signaling pathways through modifications of protein structure and enzymatic activities (Trachootham et al. 2008) and promote many aspects of cancer development.

In this study, we have provided evidence for a key role of ROS production in p38 phosphorylation probably through the activation of kinases (such as MEK3/6) that will further phosphorylate and activate p38. Our data indicate that ROS is also involved in ERK1/2

phosphorylation and activation which might be through activation of kinases (such as MEK1/2) that will phosphorylate ERK1/2. Since AKT acts as a positive regulator for IFN- γ signaling through phosphorylation and activation of STAT1 (Krämer et al. 2009; Kumatori et al. 2002), it should be activated in early stages of the pathway, but it seems that at late stages, AKT activity is down regulated by phosphatases as a negative feedback pathway. In this study, in order to overcome the high levels of ROS in MCF-7 cancer cells, high concentrations of NAC (1 mM) were needed to inhibit H_2O_2 production and the phosphorylation of STAT1, P38 and ERK1/2.

It's important to note that type I IFNs were shown to downregulate myeloid cell IFN- γ receptor by inducing

Fig. 8 Suggested model

recruitment of an early growth response 3/NGFI-A binding protein 1 complex that silences IFN- γ R1 transcription (Kearney et al. 2013). In addition, it has been revealed that IFN- γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer (Abiko et al. 2015). Recently, loss of IFN- γ pathway genes in tumor cells was demonstrated to act as a mechanism of resistance to anti-CTLA-4 therapy (Gao et al. 2016). Finally, recent work by Shi et al. showed that interdependent IL-7 and IFN- γ signaling in T-cell regulates tumour eradication by combined α -CTLA-4 + α -PD-1 therapy (Shi et al. 2016).

Our results confirmed that ROS is an important positive regulator of AKT, ERK1/2, p38 and STAT1 phosphorylation and also an activator of IFN- γ signaling pathway in an in vitro model of breast cancer cells (MCF-7). These data proved that IFN- γ signaling pathway plays important roles at early stages by inducing ROS, through H_2O_2 production, and that it follows the Src/Raf/ERK pathway, rather than the PI3K/AKT pathway. Since IFN- γ has been clinically used to treat a variety of malignancies, this study highlighted IFN- γ effects on increasing ROS production which may lead to several modifications on intracellular signaling pathways, and hence could be involved in promoting many aspects of cancer development, pathological and cytotoxic effects. Specifically inhibiting IFN- γ induced ROS production would be an interesting strategy for the treatment of breast cancer tumors. Further evaluation of

IFN- γ signaling and novel small molecule inhibitors may provide additional targeting strategies for breast cancer therapy.

In conclusion, we have shown that IFN- γ -induced ROS mediates the phosphorylation of several signaling proteins in MCF-7 cell line. Further investigations need to be performed on the major substrates that are affected by ROS activity such as kinases and phosphatases. In fact, other downstream proteins of IFN- γ signaling pathway such as STAT3, STAT5, AP1 and NF κ B need to be evaluated since they were shown to be modified in other studies (Hu and Ivashkiv 2009).

AKT/PKB, Protein kinase B; ERK, Extra Cellular Signal Regulated Kinase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; H_2O_2 , Hydrogen peroxide; INF- γ , Interferon Gamma; INF- γ R, Interferon Gamma Receptor; JAK, Janus Activated Kinase; MAPK, Mitogen Activated Protein Kinase; MEK1/2, Mitogen/ Extracellular signal regulated kinase 1/2 (also called MAPKK1/2); MEK3/6, Mitogen/Extracellular signal regulated kinase 3/6 (also called MAPKK3/6); NAC, N-acetyl cysteine; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; NF κ B, Nuclear Factor Kappa Beta; NOX, NADPH Oxidase; P38, Protein 38 kDa; PI3K, Phospho-inositide 3 Kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine; ROS, Reactive Oxygen Species; STAT, Signal Transducer and Activator of Transcription; TNF, Tumor Necrosis Factor

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Compliance with ethical standards

Conflict-of-interest The authors declare no competing financial interests.

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