Biochemical Pharmacology 82 (2011) 1145–1152

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The NADPH oxidase cytosolic component p67phox is constitutively phosphorylated in human neutrophils: Regulation by a protein tyrosine kinase, MEK1/2 and phosphatases 1/2A

Pham My-Chan Dang [a,b], Houssam Raad [a,b], Riad Arabi Derkawi [a,b], Tarek Boussetta [a,b], Marie-Hélène Paclet [d], Sahra Amel Belambri [a,b], Karama Makni-Maalej [a,b], Yolande Kroviarski [a,c], Françoise Morel [d], Marie-Anne Gougerot-Pocidalo [a,c], Jamel El-Benna [a,b,*]

[a] INSERM, U773, Centre de Recherche Biomédicale Bichat Beaujon CRB3, Paris F-75018, France
[b] Université Paris 7 site Bichat, UMRS 773, Paris F-75018, France
[c] AP-HP, Centre Hospitalier Universitaire Xavier Bichat, CIB Phenogen, Paris F-75018, France
[d] GREPI TIMC-IMAG UMR CNRS 5525, Laboratoire d'Enzymologie, Centre Hospitalier Universitaire, 38043 Grenoble, France

ARTICLE INFO

Article history:
Received 17 March 2011
Accepted 7 July 2011
Available online 20 July 2011

Keywords:
Neutrophils
NADPH oxidase
p67phox
ROS
Inflammation
Phosphorylation

ABSTRACT

Neutrophils play a key role in host defense and inflammation through the production of superoxide anion and other reactive oxygen species (ROS) by the enzyme complex NADPH oxidase. The cytosolic NADPH oxidase component, p67phox, has been shown to be phosphorylated in human neutrophils but the pathways involved in this process are largely unknown. In this study, we show that p67phox is constitutively phosphorylated in resting human neutrophils and that neutrophil stimulation with PMA further enhanced this phosphorylation. Inhibition of the constitutively active serine/threonine phosphatases type 1 and type 2A (PP1/2A) by calyculin A resulted in the enhancement of p67phox phosphorylation. Constitutive and calyculin A-induced phosphorylation of p67phox was completely inhibited by the protein tyrosine kinase inhibitor genistein and partially inhibited by the MEK1/2 inhibitor PD98059, but was unaffected by GF109203X, wortmannin and SB203580, inhibitors of PKC, PI3K and p38MAP kinase, respectively. Two-dimensional phosphopeptide mapping revealed that constitutive and calyculin A-induced p67phox phosphorylation occurred on the same major sites. Interestingly, calyculin A enhanced formyl-Met-Leu-Phe (fMLP)-induced superoxide production, while genistein inhibited this process. Taken together, these results suggest that (i) p67phox undergoes a continual cycle of phosphorylation/dephosphorylation in resting cells; (ii) p67phox phosphorylation is controlled by MEK1/2 and an upstream tyrosine kinase; (iii) PP1/2A directly or indirectly antagonize this process. Thus, these pathways could play a role in regulating ROS production by human neutrophils at inflammatory sites.

1. Introduction

Neutrophils play a key role in host innate immune responses and inflammation [1–4]. After phagocytosis of the pathogen, they produce superoxide anion, a precursor of anti-bacterial reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and hypochloric acid [3,4]. The enzyme responsible for superoxide anion production is called the NADPH oxidase [5,6]. In resting cells, the NADPH oxidase is inactive and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components (p47phox, p67phox, p40phox and Rac2) migrate to the membranes, where they associate with the membrane-bound component (flavocytochrome b558) to assemble the catalytically active oxidase [7,8]. Cytochrome b558 is a heterodimeric membranous protein composed of a large subunit (gp91phox, now called NOX2) and a small subunit (p22phox).

The predicted amino-acid sequence of p67phox contains two SH3 domains, four tetratricopeptide-rich regions (TPR), one PB1 domain and at least one proline-rich region [7,8]. P67phox associates tightly with the cytoskeleton [9–11], interacts with p40phox and p47phox in resting cells, and interacts with rac1/2 and with cytochrome b558 in activated cells [7,8]. In addition, p67phox can regulate cytochrome b558 catalytic activity by a sequence called the activation domain [12].

Upon neutrophil stimulation, p47phox [13–15], p67phox [16,17], p40phox [18] p22phox [19], and gp91phox/NOX2 [20],

* Corresponding author at: INSERM, U773, Centre de Recherche Biomédicale Bichat Beaujon (CRB3), Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, Paris F-75018, France. Tel.: +33 1 57 27 77 23; fax: +33 1 57 27 74 71.
E-mail address: jamel.elbenna@inserm.fr (J. El-Benna).

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doi:10.1016/j.bcp.2011.07.070

become phosphorylated through the engagement of a multitude of transductional pathways leading to NADPH oxidase activation [21–23]. Several stimuli, such as phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), and opsonized zymosan, induce NADPH oxidase activation in neutrophils. It is now clear that the activation of the NADPH oxidase requires the assembly of phosphorylated p47phox [24–26] and p67phox and their translocation from the cytosol to the membrane, followed by their interaction with cytochrome b558 [27,28].

The intracellular signaling pathways involved in p47phox phosphorylation have been extensively studied in human neutrophils and other cells [29–31], but those involved in p67phox phosphorylation remain to be defined. We and others have shown that p67phox can be phosphorylated on serine and threonine residues by PKC- and ERK/p38MAPK-dependent pathways in stimulated human neutrophils [16,32,33], although other protein kinases are also able to phosphorylate p67phox *in vitro* and in intact cells [34,35]. P67phox is known to be phosphorylated on threonine 233 but the phosphorylation of other sites has not been entirely ruled out [33]. The phosphorylation state of p67phox in resting neutrophil has not been clearly defined yet.

In this study, we analyzed the status of p67phox phosphorylation in neutrophils. We clearly show that p67phox is constitutively phosphorylated in resting human neutrophils, a process that was enhanced by stimulation. We further show that the constitutive phosphorylation is the net result of a balance between the activity of genistein-sensitive protein tyrosine kinases/MEK1/2 and that of PP1/2A phosphatases.

2. Materials and methods

2.1. Reagents

Phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), phenylmethylsulfonylfluoride (PMSF), Triton X-100, bovine serum albumin (BSA), Tween-20, para-nitrophenylphosphate (pNPP) and other chemicals were purchased from Sigma–Aldrich Chemical Co (Saint Louis, Missouri, USA). Dextran T500, Ficoll, [^{32}P]-orthophosphoric acid (H_3PO_4), and Gamma-bind sepharose beads were purchased from GE-Healthcare (Orsay, France). Diisopropylfluorophosphate (DFP), kinases and phosphatases inhibitors were from Calbiochem-Merck (Nottingham, UK). Endotoxin-free buffers and salt solutions were from Invitrogen (Cergy Pontoise, France). Reagents for SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), two-dimensional gel electrophoresis and Western blotting were purchased from Bio-Rad (Richmond, CA, USA). Rabbit polyclonal antibodies against the C-terminal sequence of p67phox were prepared as previously described [16,36].

2.2. Neutrophil preparation

Human neutrophils were obtained by Dextran sedimentation and Ficoll centrifugation under LPS-free conditions [16,31,32] and resuspended in phosphate-free buffer (10 mM Hepes pH 7.4, 137 mM NaCl, 5.4 mM KCl, 5.6 mM D-glucose, 0.8 mM $MgCl_2$, and 0.025% bovine serum albumin). For protein preservation, neutrophils were treated with the serine protease inhibitor diisopropylfluorophosphate (DFP) (2.7 mM) for 20 min at 4 °C before use.

2.3. Labeling of neutrophils and immunoprecipitation of p67phox and p47phox

Cells were incubated in phosphate-free buffer containing 0.5 mCi [^{32}P]-orthophosphoric acid/10^8 cells/ml for 60 min at 30 °C, as previously reported [16,32]. Neutrophils were then incubated at 37 °C for 30 min for constitutive phosphorylation, or treated with 10^{-6} M fMLP for 1 min or with 200 ng/ml PMA for 8 min. The reaction was stopped by adding ice-cold buffer, followed by centrifugation at 400 × *g* for 6 min at 4 °C. The cells were lysed by suspension in lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.25 M sucrose, and protease and phosphatase inhibitors), as previously described [16,32]. The suspension was sonicated on ice for 3 × 15 s and the lysate was centrifuged at 100,000 × *g* for 30 min at 4 °C in a TL100 ultracentrifuge (Beckman Inc.).

The cleared supernatant was incubated overnight either with anti-p67phox antibody (1/200) or with anti-p47phox antibody (1/200) and each protein was then immunoprecipitated by incubation with Gamma-bind G-sepharose beads (GE-Healthcare Orsay, France) that were then washed with lysis buffer, as previously described [16,32]. The proteins were denaturated in Laemmli sample buffer and analyzed by electrophoresis [37].

2.4. Electrophoresis and blotting

The samples were subjected to SDS-PAGE in 10% polyacrylamide gels, using standard techniques [37]. The separated proteins were electro-transferred to nitrocellulose membranes [38] and detected by means of autoradiography or Western blotting with a specific antibody as described elsewhere [16,32]. In brief, membranes were saturated for 30 min with 5% non-fat dry milk in Tris buffered saline with 0.1% tween-20 (TBST). Membranes were then probed with the anti-p67phox or p47phox antibody (1:5000 dilution) for 1 h at room temperature and washed 3 times with the TBST. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit antibodies for 1 h at room temperature (1:5000 dilution) and washed three times. Bound antibody was visualized using Enhanced chemiluminescence (ECL) (GE-Healthcare Orsay, France). Alternatively, the membranes were incubated with an alkaline phosphatase-conjugated goat-anti-mouse or goat anti-rabbit and proteins were revealed with the NBT/BCIP reagents (Saint Louis, Missouri, USA) in the carbonate buffer (0.1 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8).

2.5. Quantification of p67phox and p47phox

^{32}P-radioactivity quantification (cpm) of phosphorylated p67phox and p47phox, was determined in an Instant Imager apparatus (Packard) equipped with Instant Imager software. Radioactivity counts were corrected for protein content assessed by densitometric analysis of the intensity of the p67phox and p47phox protein bands revealed by Western blotting using the Scion Image analysis program from NIH (USA). Alternatively p67phox phosphorylation was quantified by densitometry using the same program.

2.6. Two-dimensional gel electrophoresis

Neutrophils were incubated in Hanks buffer at 37 °C for 10 min in the absence or presence of 200 ng/ml PMA for 8 min, and the reaction was then stopped by adding ice-cold PBS buffer followed by centrifugation at 400 × *g* for 6 min at 4 °C. Resting and activated neutrophils were lysed in 2-D sample buffer (9.8 M urea, 2% NP-40, 2% ampholines, 1% 2-mercaptoethanol) and analyzed by isoelectrofocusing and SDS-PAGE as previously described [39–41].

2.7. Two-dimensional tryptic phosphopeptide mapping of p67phox

Tryptic digestion of p67phox on nitrocellulose, thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC) were performed as previously described [16,32]. The nitrocellulose area

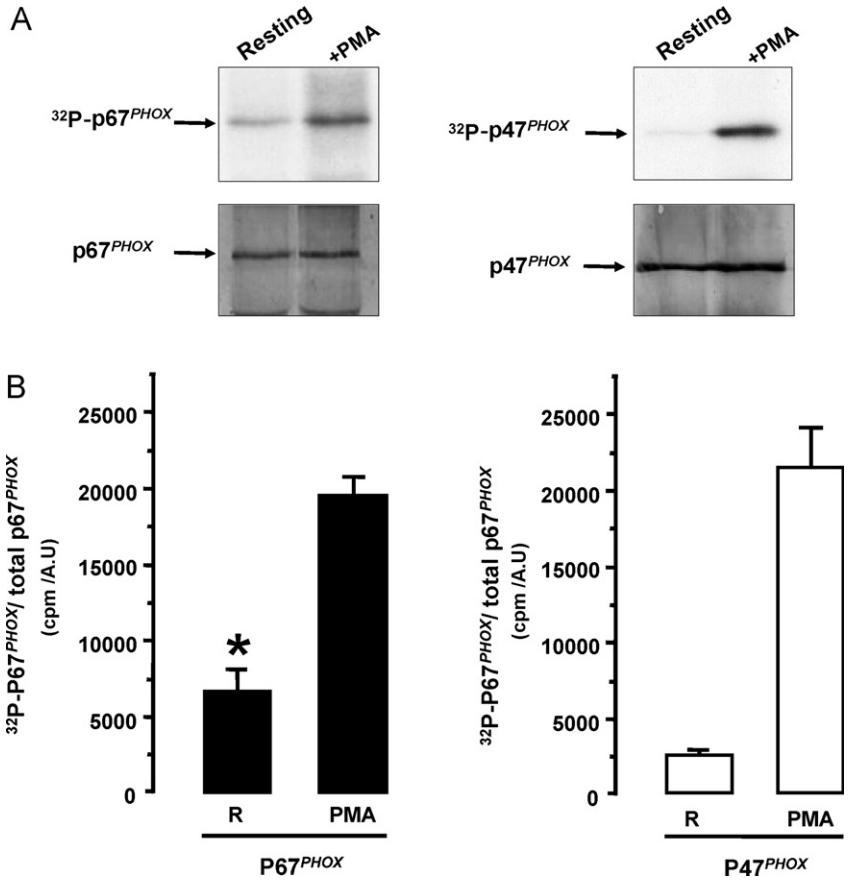

Fig. 1. Phosphorylation of p67phox and p47phox in resting and activated neutrophils. (**A**) ^{32}P-labeled neutrophils (50×10^6 cells/ml) were incubated at 37 °C (resting), then stimulated with PMA (0.2 μg/ml) for 6 min. P67phox and p47phox were immunoprecipitated and the immunoprecipitates were separated by SDS-PAGE, electro-transferred and revealed by autoradiography (^{32}P-p67phox and ^{32}P-p47phox) and immunoblotting with anti-p67phox and anti-p47phox antibodies. (**B**) Quantitative analysis of phosphorylated p67phox and p47phox proteins in human neutrophils: phosphorylated p67phox and p47phox from resting (R) or stimulated (PMA) neutrophils from different experiments were quantified by phospho-Imager analysis. Results are expressed as the ratio of radioactivity over the amount (cpm/A.U) of immunoprecipitated p67phox and p47phox determined by densitometry of the blots, as described in Section 2 (mean ± SEM, $n = 5$).

containing ^{32}P-labeled p67phox was incubated for 30 min at 37 °C with polyvinylpyrrolidone, washed, and then incubated overnight with trypsin (50 μg/ml) in carbonate buffer. Released peptides were washed three times in a Speed-Vaac, dissolved in electrophoresis buffer (17 vol water/3 vol 88% formic acid) and applied to one corner of a cellulose thin-layer plate (Merck). After electrophoresis (1000 V for 20 min), chromatography was performed as previously described [16,32]. The plates were autoradiographed for one week at −75 °C.

2.8. Statistical analysis

All results are expressed as means ± SEM. Significant differences were demonstrated using Student's t test (significance for $p < 0.05$).

3. Results

3.1. Constitutive phosphorylation of p67phox in resting human neutrophils

To study the phosphorylation of p67phox in human neutrophils, cells were labeled with ^{32}P, incubated at 37 °C in the absence (Resting) or presence of PMA (200 ng/ml for 8 min) and lysed. P67phox was immunoprecipitated from neutrophil lysates using a specific polyclonal antibody and the immunoprecipitates were analyzed by SDS-PAGE, transfer to nitrocellulose and autoradiography. The immunoprecipitated protein was further identified by the anti-p67phox antibody. As shown in Fig. 1 (left panel) p67phox was clearly phosphorylated in resting cells while less ^{32}P incorporation was observed in p47phox, which was similarly immunoprecipitated from the same lysate, (right panel). Stimulation with PMA greatly increased the phosphorylation of both proteins as previously reported [16,24]. Western blotting analysis showed equal amount of immunoprecipitated proteins for each condition. Immunoprecipitation with the control IgG showed no phosphorylated proteins by autoradiography of the gel (data not shown). Phosphorylated p67phox and p47phox from different experiments were quantified by phospho-Imager analysis and the results were expressed as the ratio of immunoprecipitated radioactivity to the amount of immunoprecipitated p67phox or p47phox that was quantified by densitometry as described in Section 2. The mean of these experiments is shown in the lower panels. Altogether, these results demonstrate that, while stimulation with PMA can increase phosphorylation of both p67phox and p47phox, only p67phox is consistently phosphorylated in resting cells. These phosphorylations were also increased by fMLP (data not shown).

To rule out the possibility that the observed constitutive phosphorylation of p67phox might occur during ^{32}P cell labeling, we compared the phosphorylation of p67phox and p47phox in the same preparation by two-dimensional gel electrophoresis and immunoblotting with anti-p67phox and anti-p47phox specific antibodies, a technique that does not require ^{32}P labeling and that we and others have used in the past [14,41]. Indeed, phosphorylation can be followed by the change in the isoelectric point of proteins that shifts to a more acidic range, giving rise to several

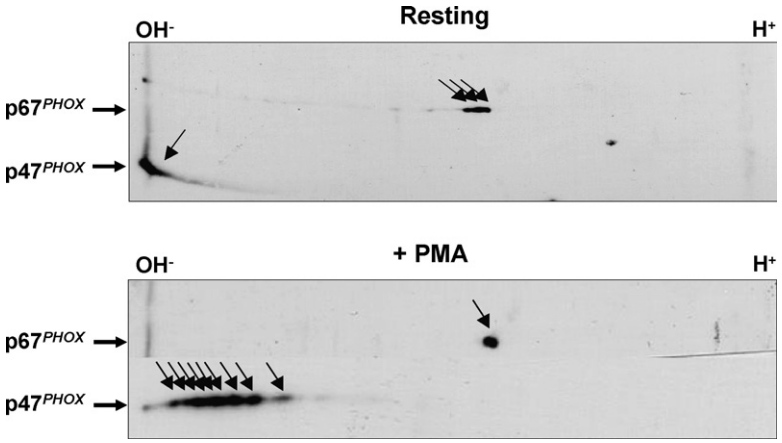

Fig. 2. Two-dimensional gel electrophoresis of resting and PMA-activated neutrophils, and Western blotting of p67phox and p47phox. Resting (top) and PMA-stimulated (bottom) neutrophils were lysed and analyzed by two dimensional gel electrophoresis and Western blotting with specific p67phox and p47phox antibodies. Data are representative of three experiments.

phosphorylated isoforms. In resting cells, p67phox migrated as three spots, indicating the presence of three isoforms (probably due to different phosphorylation states of the protein), while p47phox migrated as one very basic spot (Fig. 2), indicating the absence of phosphorylated isoform. In PMA-activated cells, p67phox slightly shifted to a very acidic isoform resulting from its complete phosphorylation while p47phox shifted to several more acidic phosphorylated isoforms, as previously demonstrated [14,41]. Thus, these results confirm those obtained with ^{32}P labeling, demonstrating that, in contrast to p47phox, p67phox is constitutively and partially phosphorylated in resting human neutrophils.

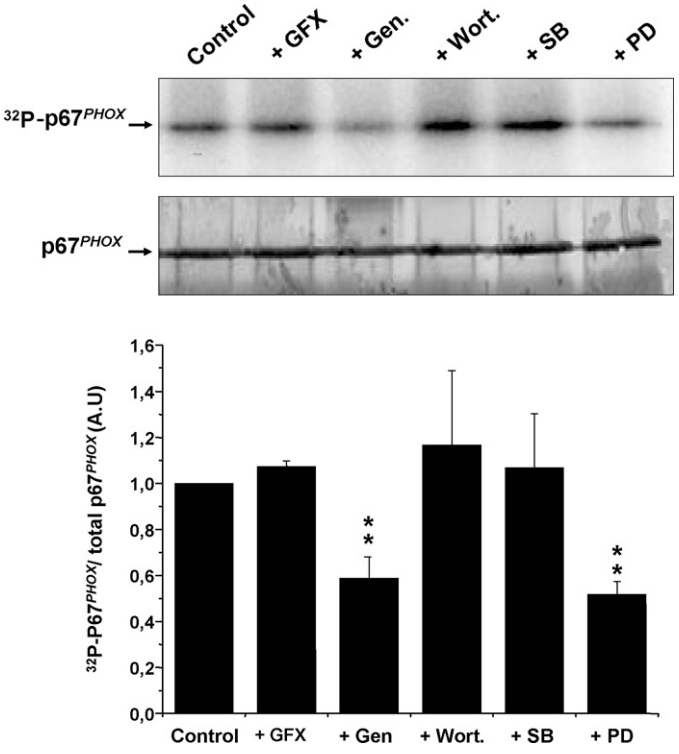

Fig. 3. Effect of protein kinase inhibitors on constitutive p67phox phosphorylation. ^{32}P-labeled neutrophils (50×10^6 cells/ml) were incubated in the presence or absence of GFX109203X (GFX; 5 μM), genistein (Gen.; 100 μM), Wortmannin (Wort.; 100 nM), SB203580 (SB; 10 μM) and PD98059 (PD; 50 μM) at 37 °C for 30 min. P67phox was immunoprecipitated and the immunoprecipitates were analyzed by SDS-PAGE, followed by electro-transfer and autoradiography (^{32}P-p67phox) or Western blotting with anti-p67phox antibody. Quantitative analysis of phosphorylated p67phox proteins in human neutrophils (lower panel): phosphorylated p67phox and total p67phox from different experiments were quantified by densitometry of the film and the blot respectively. Results are expressed as the ratio of phosphorylated over the amount of immunoprecipitated p67phox (A.U/A.U) (mean ± SEM, $n = 3$) (** $p < 0.05$ compared to control).

3.2. Constitutive p67phox phosphorylation is controlled by a genistein-sensitive tyrosine kinase and MEK1/2

To understand the pathways involved in constitutive p67phox phosphorylation, ^{32}P-labeled neutrophils were incubated with different protein kinase inhibitors and p67phox phosphorylation was analyzed as described above. Preincubation of neutrophils with the tyrosine kinase inhibitor genistein inhibited the constitutive phosphorylation of p67phox (Fig. 3). In contrast, preincubation of neutrophils with the PKC inhibitor GF109203X, or the PI3K inhibitor wortmannin, or the p38MAPKinase inhibitor SB203580 did not inhibit p67phox phosphorylation in resting cells. The MEK1/2 inhibitor PD98059 only partially reduced p67phox phosphorylation.

3.3. Inhibition of the constitutive active phosphatase PP1/2A by calyculin A resulted in the enhancement of p67phox phosphorylation, a process controlled by a tyrosine kinase and MEK1/2

Phosphorylation is a dynamic process and the phosphorylation state of proteins at any point in time is the result of a balance between the activities of kinases and phosphatases. The results obtained above showing that incubation of resting neutrophils with genistein inhibits p67phox phosphorylation suggests that in resting cells when a tyrosine kinase is inhibited, p67phox is dephosphorylated over time by an active phosphatase. Thus, to determine the potential role of phosphatases in the constitutive phosphorylation of p67phox in resting neutrophils, ^{32}P-labeled neutrophils were treated with calyculin A, an inhibitor of phosphatase 1 and phosphatase 2A (PP1/2A). Results show (Fig. 4) that calyculin A induced a time- and concentration-dependent increase in p67phox phosphorylation. These results suggest that PP1/2A may regulate the phosphorylation of p67phox in resting cells.

To further characterize the constitutive phosphorylation of p67phox, we performed tryptic phosphopeptide maps of p67phox isolated from resting and calyculin A-treated neutrophils. Fig. 5 shows that in resting neutrophils, p67phox is phosphorylated on one major peptide and one minor peptide (designated peptides a and b) Calyculin A induced the phosphorylation of one major

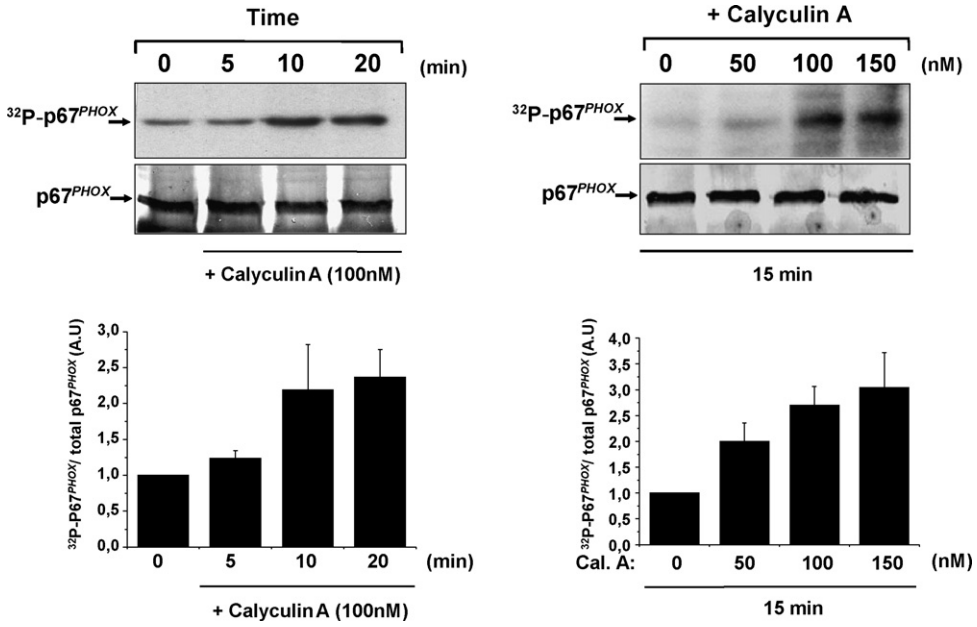

Fig. 4. Effect of the phosphatase 1/2A inhibitor calyculin A on p67phox phosphorylation. ^{32}P-labeled neutrophils (50×10^6 cells/ml) were incubated in the presence or absence of calyculin A at 37 °C for indicated times and concentrations. P67phox was immunoprecipitated and the immunoprecipitates were analyzed by SDS-PAGE, followed by electro-transfer and autoradiography (^{32}P-p67phox). Quantitative analysis of phosphorylated p67phox proteins in human neutrophils (lower panel): phosphorylated p67phox and total p67phox from different experiments were quantified by densitometry of the film and the blot respectively. Results are expressed as the ratio of phosphorylated over the amount of immunoprecipitated p67phox (A.U/A.U) (mean ± SEM, $n = 3$).

peptide (peptide a) and two minor peptides (peptides b and c). Taken together, these results suggest that p67phox is constitutively phosphorylated on its major sites and these sites are targeted by phosphatases PP1/2A.

To further check if the genistein-sensitive tyrosine kinase controls calyculin A-induced p67phox phosphorylation, we tested the effect of genistein on this process. Preincubation of ^{32}P-labeled neutrophils (15 min) with genistein completely inhibited the calyculin A-induced phosphorylation of p67phox (Fig. 6). The effect of genistein was concentration dependent starting at 25 μM (Supplementary data 1). However, preincubation of neutrophils with the PKC inhibitor GF109203X, the PI3K inhibitor wortmannin, and the p38MAPKinase inhibitor SB203580 did not result in inhibition of calyculin A-induced p67phox phosphorylation. MEK1/2 inhibitor PD98059, partially inhibited calyculin A-induced p67phox phosphorylation. Western blotting analysis showed equal amount of immunoprecipitated proteins for each condition. These data strongly suggest that p67phox undergoes a continual cycle of phosphorylation/dephosphorylation in resting cells controlled by a genistein-inhibitable tyrosine kinase, MEK1/2 and by the PP1/2A phosphatases.

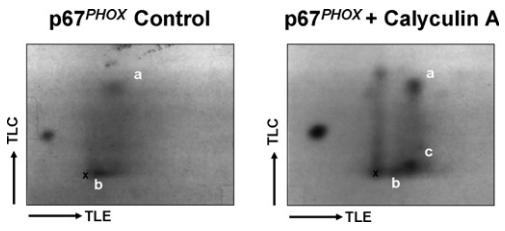

Fig. 5. Two-dimensional phosphopeptide mapping of constitutive and calyculin A-induced p67phox phosphorylation. Phosphorylated p67phox isolated from resting and calyculin A-treated neutrophils was cleaved by trypsin and analyzed by thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC). The phosphopeptides were detected by autoradiography. Peptides are designated: a, b and c. Data are representative of three experiments.

Human neutrophils, express several tyrosine kinases such as Lyn, Syk and JAK [42–48]. In order to identify the protein tyrosine kinase involved in p67phox phosphorylation, we have tested the effect AG490 a JAK2 inhibitor, PP1 a src tyrosine kinase inhibitor and Piceatannol a syk inhibitor on the phosphorylation of p67phox. Results show (Fig. 7), that although genistein completely inhibited Calyculin A-induced p67phox phosphorylation, none of the inhibitors tested at a concentration known to inhibit the corresponding kinase had a clear effect.

3.4. Inhibition of the constitutively active phosphatase PP1/2A by calyculin A resulted in the enhancement of superoxide production, a process inhibited by genistein

Inhibition of the phosphatase PP1/2A by calyculin A resulted in the enhancement of fMLP-induced superoxide production due to NADPH oxidase upregulation in human neutrophils. As genistein completely inhibited calyculin A-induced p67phox phosphorylation, we wanted to see if this pathway is involved in calyculin A-induced NADPH oxidase upregulation.

Our results show that in resting conditions and in the presence of calyculin A alone, neutrophils produced a basal low level of superoxide as measured by cytochrome c reduction assay (Fig. 8) and genistein inhibited this response. FMLP alone stimulated superoxide production. As expected, subsequent addition of fMLP to neutrophils exposed to calyculin A resulted in increased superoxide production. Treatment of neutrophils with genistein, resulted in the inhibition of fMLP-induced superoxide production and more interestingly inhibited the enhancing effect induced by calyculin A.

4. Discussion

Unlike p47phox phosphorylation, p67phox phosphorylation has not been extensively investigated. We [16,32] and others [33–35] have shown that p67phox becomes phosphorylated upon stimulation of human neutrophils, monocytes and lymphoblasts

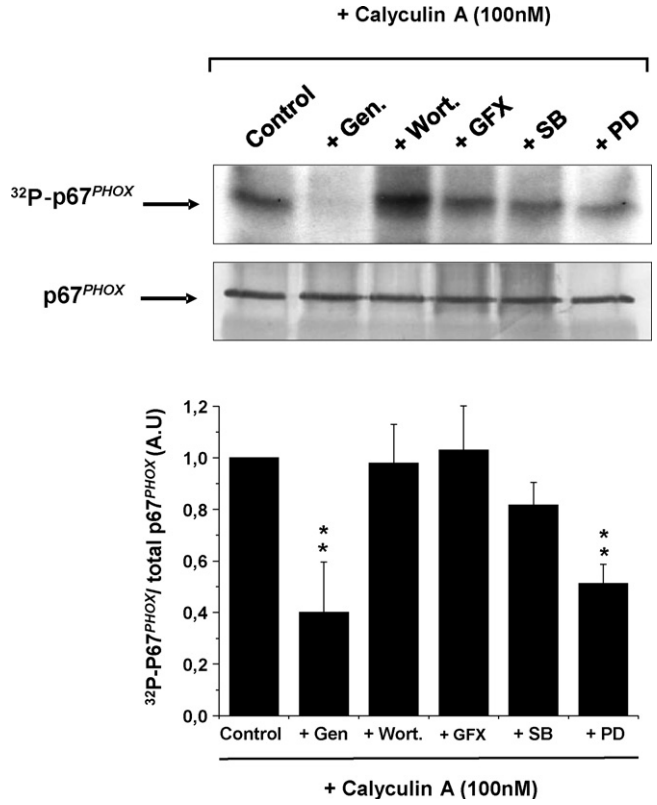

Fig. 6. Effect of protein kinase inhibitors on calyculin A induced-p67phox phosphorylation. ^{32}P-labeled neutrophils (50×10^6 cells/ml) were incubated in the presence or absence of genistein (Gen.; 100 μM), Wortmannin (Wort.; 100 nM), GFX109203X (GFX; 5 μM), SB203580 (SB; 10 μM) and PD98059 (PD; 50 μM) at 37 °C for 15 min then cells were treated by calyculin A. P67phox was immunoprecipitated and the immunoprecipitates were analyzed by SDS-PAGE, followed by electro-transfer and autoradiography (^{32}P-p67phox). Quantitative analysis of phosphorylated p67phox proteins in human neutrophils (lower panel): phosphorylated p67phox and total p67phox from different experiments were quantified by densitometry of the film and the blot respectively. Results are expressed as the ratio of phosphorylated over the amount of immunoprecipitated p67phox (A.U/A.U) (mean ± SEM, $n = 3$) ($^{**}p < 0.05$ compared to control).

but the characteristics of this phosphorylation and the pathways involved in this process are largely unknown. In the course of our previous studies, we had noted incorporation of ^{32}P into p67phox from resting cells [16]. Thus, we decided to further investigate this process. In this study, we clearly show that, contrary to p47phox, p67phox is constitutively phosphorylated in resting human neutrophils and that this phosphorylation is further enhanced

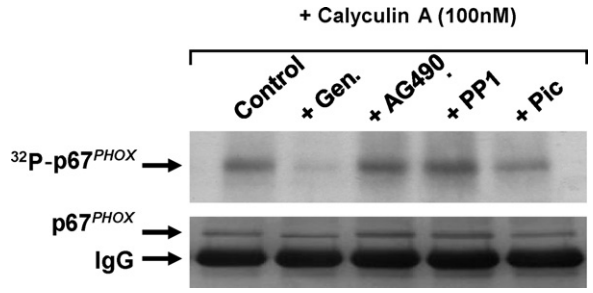

Fig. 7. Effect of protein tyrosine kinase inhibitors on calyculin A induced-p67phox phosphorylation. ^{32}P-labeled neutrophils (50×10^6 cells/ml) were incubated for 30 min in the presence or absence of genistein (Gen.; 100 μM), AG490 (100 μM), PP1 (5 μM) or Piceatannol (50 μM), incubated with 100 ng/ml of Calyculin A, lyzed and p67phox was immunprecipitated by a specific antibody. P67phox was analyzed by SDS-PAGE transfer autoradiography (^{32}P-p67phox) and Western blot (p67phox). Data are representative of three experiments.

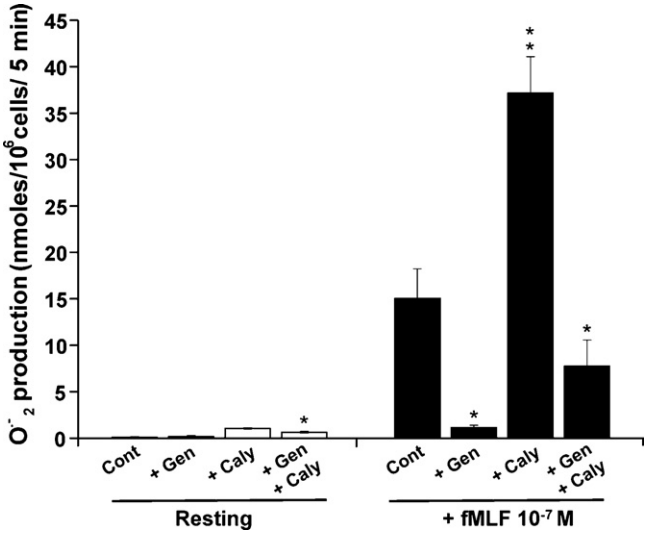

Fig. 8. Effect of calyculin A and genistein on superoxide production by human neutrophils. Neutrophils (1×10^6 cells/ml) were incubated in the presence or absence of genistein (Gen) (50 μM) or calyculin A (Caly) (100 nM) or both then stimulated by fMLP (10^{-7} M). Superoxide production was measured by the SOD-inhibitable cytochrome c reduction assay. Data are representative of three experiments ($^{*}p < 0.01$ for genistein compared to control, $^{**}p < 0.05$ for calyculin A + fMLP compared to fMLP alone).

by cell stimulation with PMA or fMLP (data not shown). In addition, we show that constitutive p67phox phosphorylation is controlled by a tyrosine kinase-dependent pathway and by PP1/2A phosphatases.

The basal p67phox phosphorylation observed here was constitutive, and was not due to stimulation during cell handling, as p47phox, isolated from the same lysates, was not phosphorylated, even though it possesses several serines that could be phosphorylated [15,24]. The two-dimensional gel electrophoresis and immunoblotting experiments further confirmed the constitutive nature of the phosphorylation, and suggested that at least two phosphorylated isoforms were present under basal conditions, indicating that p67phox could be phosphorylated on at least two different residues. In keeping with these data, the two-dimensional phosphopeptide mapping showed that p67phox isolated from resting neutrophils migrated as one major phosphorylated peptide and one minor peptide. Calyculin A enhanced the phosphorylation of these peptides. These results suggest that p67phox is constitutively phosphorylated on its major and two minor sites, and that these sites are targeted by phosphatases PP1/2A. At this point, it is unclear whether the sites phosphorylated in resting neutrophils are identical to those identified in stimulated cells. Forbes et al. [33] reported that threonine 233, which is located in a proline-rich region, is a major phosphorylated site for p67phox isolated from stimulated neutrophils and we previously demonstrated that fMLP and PMA induce phosphorylation of p67phox on serine residues [16].

We have previously shown that p67phox is phosphorylated by a PKC-dependent pathway in fMLP- and PMA-stimulated human neutrophils [12,32] and others have shown that PKCdelta phosphorylates p67phox in opsonized-zymosan stimulated monocytes [35]. PKC does not appear to be involved in the constitutive phosphorylation of p67phox as the general PKC inhibitor GF109203X had no effect, although it inhibited PMA-induced p47phox phosphorylation (Supplemental Data 2). We also reported that ERK1/2 and p38MAPK phosphorylated p67phox *in vitro* and in fMLP- and PMA-stimulated neutrophils [32]; however, only ERK1/2 are involved in constitutive p67phox phosphorylation since PD98059, the MEK1/2 inhibitor, partially inhibited p67phox phosphorylation.

Our previous studies demonstrated the involvement of a PKC-independent pathway in p67phox phosphorylation [16,32]. Here, we provide evidence that a protein tyrosine kinase-dependent pathway is involved in basal phosphorylation of p67phox. It is well known that genistein inhibits superoxide production in fMLP-stimulated neutrophils [42,43], and prevents rac translocation [44]. Our data add p67phox as a target for a protein tyrosine kinase-dependent pathway. Interestingly, PMA-induced phosphorylation was not inhibited by genistein, consistent with the lack of effect of genistein on PMA-stimulated superoxide production [45]. Several tyrosine kinases are expressed in neutrophils, such as Lyn, Syk and JAK, and they are activated by fMLP and other stimuli in human neutrophils [46–49]. In order to identify the protein tyrosine kinase involved in p67phox phosphorylation, we have tested the effect AG490 a JAK2 inhibitor, PP1 a src tyrosine kinase inhibitor and Piceatannol a syk inhibitor on the phosphorylation of p67phox. Results showed that although genistein completely inhibited Calyculin A-induced p67phox phosphorylation, none of the inhibitors tested had a comparable effect. One possible explanation is that an other protein tyrosine kinase is involved in this process or that genistein could have a non-specific effect at the concentration used here. Genistein was not toxic at the concentrations tested but it could inhibit other targets. More investigation will be necessary to identify the protein tyrosine kinase involved. However these tyrosine kinases might not directly phosphorylate p67phox but rather regulate downstream kinases such as ERK1/2.

It is well known that calyculin A potentiates the respiratory burst of neutrophils and macrophages [44,50–54]. Calyculin A was also shown to enhance the translocation to membranes of all cytosolic components of the NADPH oxidase [44] and to increase and prolong the phosphorylation of p47phox in stimulated cells [51,55]. Here, we show that calyculin A enhanced the constitutive phosphorylation of p67phox and NADPH oxidase activation, and genistein inhibited both process, suggesting that phosphorylated p67phox is a target for PP1/PP2A and this pathway could regulate NADPH oxidase activation.

How this constitutive phosphorylation of p67phox could regulate NADPH oxidase activation is not known. This phosphorylation could have a role in maintaining the oxidase inactive in the cytosol or it could prepare p67phox to a better activation of this oxidase. This phosphorylation did not alter binding of p67phox to the p40phox and p47phox complex (data not shown). The fact that p67phox is phosphorylated on the same sites in resting and in PMA and fMLP-stimulated neutrophils [16,32], and the fact that p67phox phosphorylation was found in plasma membranes [17] suggest that this phosphorylation rather plays a positive role in activating cytochrome b 558. Identification of the phosphorylated sites, site directed mutagenesis of these sites and transfection in p67phox-deficient cells will allow us to answer this question in the future.

In summary, we demonstrated that p67phox is constitutively phosphorylated in resting human neutrophils and that the state of p67phox phosphorylation is controlled by an upstream tyrosine kinase, MEK1/2 and the serine/threonine phosphatases PP1/2A. Dysregulation of the balance between these two activities could play a role in ROS production by human neutrophils at inflammatory sites.

Acknowledgments

This work was supported by INSERM (Institut National de la Santé Et de la Recherche Médicale), CNRS (Centre National de la Recherche Scientifique), ARC (Association pour la Recherche sur le Cancer). Houssam Raad is a recipient of the FRM (Fondation pour la Recherche Médicale) fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.07.070.

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