Increased reactive oxygen species production and p47phox phosphorylation in neutrophils from myeloproliferative disorders patients with *JAK2* (V617F) mutation

Margarita Hurtado-Nedelec*,[1,2,3] Marie-José Csillag-Grange*,[3] Tarek Boussetta,[1,2] Sahra Amel Belambri,[6] Michèle Fay,[1,2] Bruno Cassinat,[4,5] Marie-Anne Gougerot-Pocidalo,[1,2,3] Pham My-Chan Dang,[1,2] and Jamel El-Benna[1,2]

[1]INSERM U773, Centre de Recherche Biomédicale Bichat Beaujon CRB3, Paris France; [2]Université Paris 7 Denis Diderot, Sorbonne Paris Cité, Laboratoire d'excellence Inflamex, Faculté de Médecine, site Bichat, Paris, France; [3]AP-HP, Unité Dysfonctionnement Immunitaire, Centre Hospitalo-Universitaire Xavier Bichat, Paris, HUPNVS; [4]AP-HP, unité de Biologie Cellulaire; [5]Inserm UMRS 940, Hôpital Saint-Louis, Paris France, Groupe PV-Nord; and [6]Laboratoire de Biochimie Appliquée, Equipe de recherche Stress oxydatif et inflammation, Département de Biologie, Faculté des Sciences, Université Ferhat Abbas, Sétif, Algeria

ABSTRACT

Myeloproliferative disorders are associated with increased risk of thrombosis and vascular complications. The pathogenesis of these complications is not completely known. Reactive oxygen species produced by the neutrophil NADPH oxidase could have a role in this process. The aim of this study was to evaluate reactive oxygen species production by neutrophils of myeloproliferative disorder patients. Patients with or without the *JAK2* V617F mutation were characterized. Reactive oxygen species production was assessed by chemiluminescence, and phosphorylation of the NADPH oxidase subunit p47phox was analyzed by Western blots. In a comparison of controls and myeloproliferative disorder patients without the *JAK2* V617F mutation, reactive oxygen species production by neutrophils from patients with the *JAK2* V617F mutation was dramatically increased in non-stimulated and in stimulated conditions. This increase was associated with increased phosphorylation of the p47phox on Ser345 and of the uspstream kinase ERK1/2. In neutrophils from healthy donors, JAK2 can be activated by GM-CSF. GM-CSF-induced p47phox phosphorylation and priming of reactive oxygen species production are inhibited by the selective JAK2 inhibitors AG490 and lestaurtinib (CEP-701), supporting a role for JAK2 in the upregulation of NADPH oxidase activation. These findings show an increase in reactive oxygen species production and p47phox phosphorylation in neutrophils from myeloproliferative disorder patients with the *JAK2* V617F mutation, and demonstrate that JAK2 is involved in GM-CSF-induced NADPH oxidase hyperactivation. As neutrophil hyperactivation could be implicated in the thrombophilic status of patients with myeloproliferative disorders, aberrant activation of JAK2 V617F, leading to excessive neutrophil reactive oxygen species production might play a role in this setting.

Introduction

Myeloproliferative disorders (MPD) such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) arise from an acquired stem cell alteration leading to abnormal production of red blood cells, thrombocytes and leukocytes.[1] A mutation in the gene encoding Janus kinase 2 (JAK2), which is involved in hematopoietic growth factor signaling, has been found in patients with BCR-ABL1-negative MPD.[2,3] Almost all patients with PV and about half those with ET have the same *JAK2* mutation V617F. This mutation results in a gain of function and induces constitutive tyrosine kinase activity.[4] *JAK2* V617F has been implicated in elevated leukocyte phosphatase activity, a marker of neutrophil activation and a pathological feature of MPD.[5-8] Arterial and venous thrombosis are a major cause of mortality of patients with MPD.[9] However, the pathogenesis of these complications is not completely known. Most of the studies into this have focused on red blood cells and platelets and the role of neutrophils has not received so much attention despite the fact that MPD patients have high numbers. Neutrophil-derived reactive oxygen species (ROS) can induce endothelial cell injury and modify the functions they have in thromboregulation.[10-13] Neutrophils have a vital role in host defenses against invading microorganisms.[14] In response to a variety of agents, they produce and release large quantities of superoxide anion (O_{2}.-) and other ROS, a process called the respiratory burst.[14,15] Production of superoxide anion is dependent on NADPH oxidase, a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to superoxide anion.[16,17]

In resting cells, NADPH oxidase is inactive and its components are distributed between the cytosol and membranes. When cells are stimulated, the cytosolic components (p47phox, p67phox, p40phox and Rac2) migrate to the membranes where they associate with the membrane-bound component (gp91phox/NOX2 and p22phox, which together form the flavocytochrome b558) to assemble the catalytically active oxidase.[18] P47phox phosphorylation on several serines plays a pivotal role in oxidase activation in intact cells.[19]

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Correspondence: jamel.elbenna@inserm.fr

Neutrophil superoxide production can be potentiated by prior exposure to 'priming' agents such as the pro-inflammatory cytokines GM-CSF, TNFα and IL-8 and LPS.[18,20,21] This process is believed to enhance ROS production at sites of infection and inflammation.

We have previously shown that GM-CSF and TNFα induce p47phox phosphorylation on Ser345 by ERK1/2 and p38MAPKinase, respectively, and that this process is involved in the priming of the neutrophil respiratory burst.[20,21] Therefore, the aim of this study was to examine ROS production by neutrophils from MPD patients with or without the *JAK2* V617F mutation compared to ROS production by neutrophils from healthy volunteers. We show that neutrophils isolated from blood of MPD patients with the *JAK2* V617F mutation display ROS hyperproduction and increased Ser345 phosphorylation on p47phox. Phosphorylation of p47phox on Ser345 and priming of ROS production induced by GM-CSF, a cytokine known to activate JAK2, are inhibited by selective JAK2 inhibitors, further supporting the link between JAK2 and NADPH oxidase.

Table 1. Characteristics of patients with myeloproliferative disorders.

	ET	PV	PMF	Neutrophilic leukemia
N. patients (Men/Women)	14 (6/8)	12 (9/3)	2 (0/2)	1 (1/0)
Age, years, median (range)	68 (35-88)	74 (49-82)	55-80	87
Hemoglobin level, g/L, median (range)	136 (101-146)	170 (140-193)	88-95	178
Hematocrit, % median (range)	39.4 (32.5-43.6)	51.4 (42.4-59.0)	30-31	54.5
WBC count x 10^9/L median (range)	8.8 (5.9-13.0)	8.4 (5.5-16.1)	11.3-18.5	46.0
PMN count x 10^9/L median (range)	5.1 (2.9-8.9)	6.4 (2.7-12.6)	8.5-12.0	38.2
PLT counts (10^9/L, median (range)	794 (533-1691)	469 (158-1100)	74-272	85
JAK2 (V617F) mutation				
N. of patients with mutation	7	10	2	1
% mutant alleles median (range)	30 (15-38)	40 (10-75)	41-80	80

ET: essential thrombocytosis; PV: polycythemia vera; PMF: primary myelofibrosis and neutrophilic leukemia.

Methods

Ethics

Neutrophils were isolated from venous blood of healthy volunteers and patients with BCR-ABL1-negative chronic MPD managed in the hematology and immunology department of Bichat Hospital, Paris, France. The investigations were approved by the local ethics committee and samples were obtained with the volunteers' and patients' written informed consent. All experiments were approved by the Institut National de la Santé et de Recherche Médicale (INSERM) institutional review board and ethics committee. Data collection and analyses were performed anonymously.

Neutrophil preparation

Circulating neutrophils were isolated by Polymorphprep gradient centrifugation.[20,21] Briefly, blood was diluted 2-fold in sterile PBS and cells were isolated by one-step Polymorphprep gradient centrifugation at 500 g for 30 min at 22°C. The neutrophil band was collected and the cells were washed in PBS and counted.

Patients and diagnostics

We studied 29 patients with BCR-ABL1-negative chronic myeloproliferative disorders managed at the hematology and immunology department of Bichat Hospital, Paris, France. Based on WHO diagnostic criteria, 14 patients had ET (7 *JAK2* V617F), 12 PV (10 *JAK2* V617F), 2 PMF (2 *JAK2* V617F), and one neutrophilic leukemia (*JAK2* V617F). *JAK2* V617F was detected and quantified by using allele-specific PCR, as previously described.[22] Patients' characteristics are summarized in Table 1.

Measurement of ROS and superoxide production

A chemiluminescence method was used to measure ROS production.[20,21] Superoxide production was measured by the superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction assay.[20,21]

Flow cytometric analysis of CD11b expression

Whole-blood samples (500 µl) were incubated at 37°C for 5 min with DPBS (resting) or fMLF (10^{-7} M). Then 100 µl of each sample were incubated with 10 µl of a PE-conjugated anti-human CD11b and 10 µl of FITC-conjugated anti-human CD15 monoclonal antibodies (BD Biosciences, San Jose, CA, USA) for 20 min at room temperature in the dark. Red blood cells were lysed with BD FACS™ lysing solution (BD Biosciences) and white blood cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry analysis.

P47phox and ERK1/2 phosphorylation in neutrophils

Neutrophils from MPD patients were lysed without stimulation. Neutrophils from healthy donors were treated with kinase inhibitors for 30 min, then with GM-CSF (12 ng/mL) at 37°C for 20 min. The reaction was stopped by adding ice-cold buffer and by centrifugation at 400 g for 6 min at 4°C. The cells were lysed by resuspension in lysis buffer. Proteins in the cleared supernatant were denatured in Laemmli's sample buffer. The samples were then subjected to SDS-10% PAGE and Western blot using standard techniques.[23,24] The intensity of phosphorylated-p47phox, total p47phox, phosphorylated-ERK and total ERK bands were quantified by densitometry using the Image J analysis program. Phosphorylated intensities were corrected for the corresponding amounts of total protein present on the membrane.

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM). Significant differences ($P<0.05$) were identified with Student's t-test.

Further information concerning Methods is available in the *Online Supplementary Appendix*.

Results

ROS production is highly increased in neutrophils isolated from patients with myeloproliferative disorders and associates with the JAK2 V617F mutation

To study ROS production by neutrophils from MPD patients, neutrophils were isolated from healthy donors as controls and from 29 patients with BCR-ABL1-negative chronic MPD managed in our hematology and immunology department. Based on WHO diagnostic criteria, 14

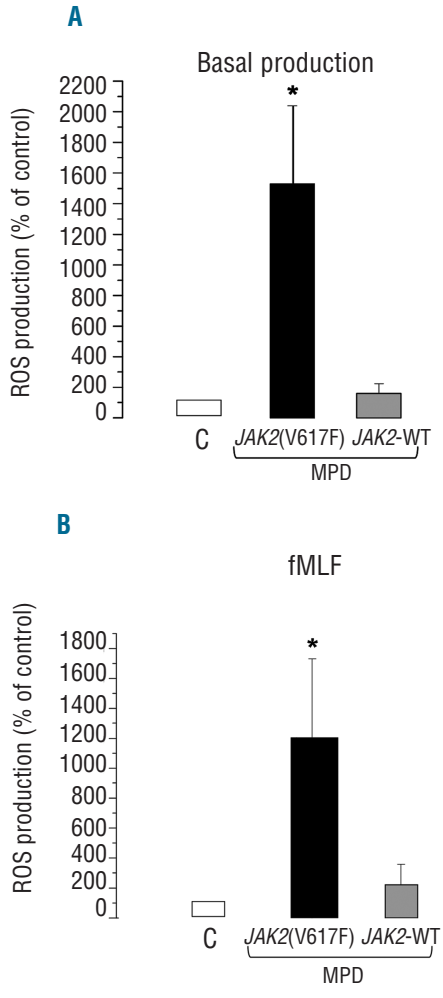

Figure 1. Basal and fMLF-induced ROS production by neutrophils from controls and patients with myeloproliferative disorders with or without the *JAK2* V617F mutation. Neutrophils (5 x 10^5 cells) isolated from blood of healthy donors (C: control) and from MPD patients with (*JAK2* (V617F)) or without the *JAK2* mutation (*JAK2* wild type: *JAK2*-WT) were incubated in HBSS in the presence of luminol (10 μM). (A) Basal ROS production was measured by luminol-amplified chemiluminescence for 15 min. Total counts were calculated and compared to control neutrophils (designated as 100 % which correspond to 7.1 ± 1.6 x 10^6 cpm). (B) The same preparation was then stimulated with fMLF (10^{-7} M), chemiluminescence was measured during 15 min and total counts were calculated and compared to control neutrophils (designated as 100 % which correspond to 57.4 ± 8.8 x 10^6 cpm). (mean ±SEM, Controls (n=18), MPD *JAK2*(V617F) (n=20), MPD *JAK2*-WT (n = 9); **P*<0.01 *versus* control).

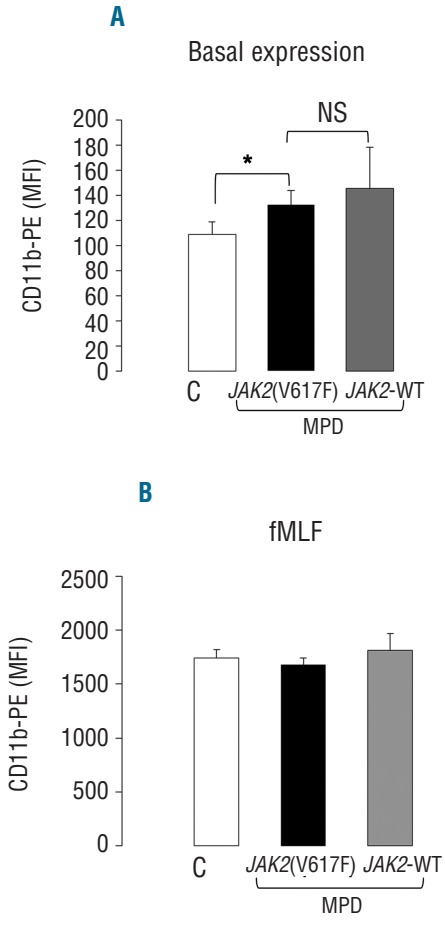

Figure 2. Basal and fMLF-induced CD11b expression by neutrophils from controls and patients with myeloproliferative disorders with or without the *JAK2* V617F mutation. (A) Whole-blood samples (500 μL) of healthy donors (C: control) and from MPD patients with (*JAK2* (V617F) or without the *JAK2* mutation (*JAK2* wild type: *JAK2*-WT) were incubated at 37 °C for 5 min without stimulation (Basal expression). Samples were incubated with a PE-conjugated anti-human CD11b monoclonal antibody for 20 min at room temperature in the dark. Red cells were lysed and white cells were resuspended in 1% paraformaldehyde-PBS and analyzed by flow cytometry. (B) The same as A except that fMLF (10^{-7} M) was used. (mean ± SEM, controls (n=18), MPD *JAK2*(V617F) (n=20), MPD *JAK2*-WT (n=9), **P*<0.05 *versus* control, NS: not significant).

patients had essential thrombocythemia (ET), 12 polycythemia vera (PV), 2 primary myelofibrosis (PMF) and one neutrophilic leukemia. *JAK2* V617F was detected and quantified by using allele-specific PCR, as previously described.[22] Among the 14 patients with ET, 7 have the *JAK2* V617F mutation. Among the 12 patients with PV, 10 have the *JAK2* V617F mutation. The 2 patients with PMF have the *JAK2* V617F mutation, and the patient with neutrophilic leukemia has the *JAK2* V617F mutation. MPD patients with or without *JAK2* V617F mutation were treated with acetylsalicylic acid and/or hydroxyurea only. The healthy volunteers were age- and gender-matched with the patients. Detailed patients' characteristics are summarized in Table 1.

Reactive oxygen species production was assessed by using luminol-amplified chemiluminescence. The results show that neutrophils from MPD patients with the *JAK2* V617F mutation had significantly higher basal ROS production than neutrophils from healthy donors and MPD patients without *JAK2* mutation (Figure 1A). Incubation of patients' neutrophils with the NADPH oxidase inhibitors diphenylene iodonium (DPI) (10 μM) or with apocynin (100 μM) for 15 min resulted in a strong inhibition of this basal ROS production (2.8 +/- 0.8% of the control (n=4; $P<0.01$) for DPI and 1.4 +/- 0.6% of the control (n=4; $P<0.01$) for apocynin). When stimulated with fMLF, neutrophils from MPD patients with the *JAK2* V617F mutation also exhibited significantly higher ROS production

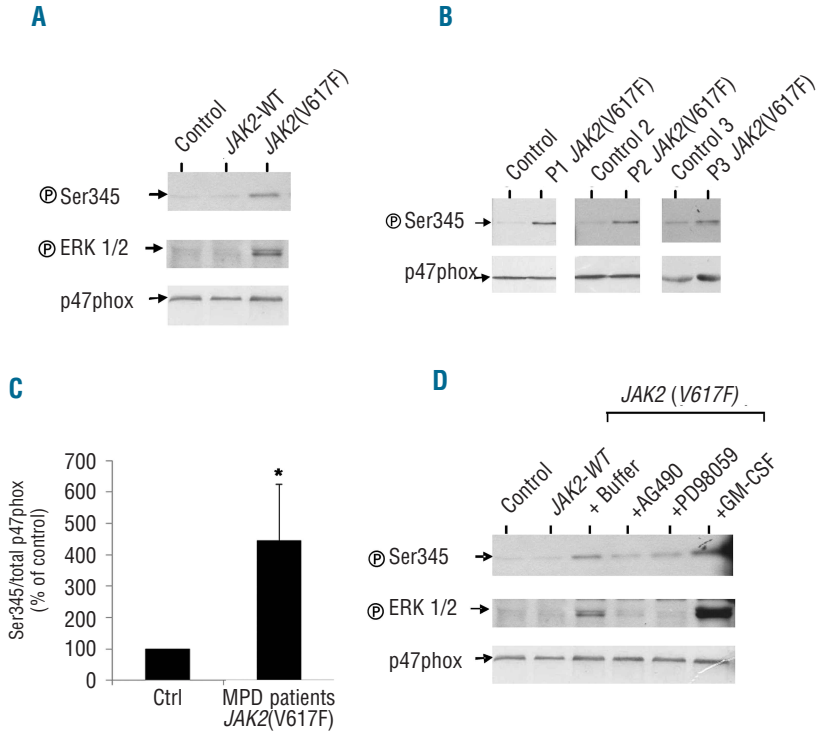

Figure 3. P47phox phosphorylation on Ser345 and phosphorylation of ERK1/2 are up-regulated in neutrophils from patients with myeloproliferative disorders associated with the *JAK2* V617F mutation. (A) Neutrophils (5 x 10^5 cells) isolated the same day, from blood of healthy donor (Control) or MPD patients without (*JAK2*-WT) or with (*JAK2*(V617F)) the *JAK2* V617F mutation, were lysed and proteins were analyzed with SDS-PAGE and immunoblotting with anti-phospho-Ser345 (pSer345) or anti-phospho-ERK1/2 or anti-p47phox. (experiments performed the same day n=5). (B) Neutrophils (5 x 10^5 cells) isolated from blood of healthy donors (Control) or MPD patients with (*JAK2*(V617F)) mutation were lysed and proteins were analyzed with SDS-PAGE and immunoblotting with anti-phospho-Ser345 (pSer345) or anti-p47phox. (C) Blots were quantified and compared. Scale bars represent means ± SEM (n = 10, **P*<0.05). (D) Neutrophils (5 x 10^5 cells) isolated the same day, from blood of healthy donor (Control) or MPD patients without (*JAK2*-WT) or with the *JAK2* V617F mutation (*JAK2*(V617F) incubated with buffer alone, 100 μM AG490, 50 μM PD98059 or 20 ng/mL GM-CSF, then lyzed and proteins were analyzed by SDS-PAGE and immunoblotting with anti-phospho-Ser345 (pSer345) or anti-phospho-ERK1/2 or anti-p47phox. (experiments performed the same day n=3).

than neutrophils from healthy donors (Figure 1B). Incubation of patients' neutrophils with the NADPH oxidase inhibitors DPI (10 μM) or with apocynin (100 μM) for 15 min resulted in strong inhibition of fMLF-induced ROS production (2.1 +/- 0.7% of the control (n=4; *P*<0.01) for DPI and 1.25 +/- 0.5% of the control (n=4; *P*<0.01) for apocynin. These results suggest that NADPH oxidase, the enzyme responsible for ROS production in neutrophils, is constitutively up-regulated or primed in MPD patients with the *JAK2* V617F mutation.

Interestingly, plasma-membrane CD11b expression, which reflects degranulation (another neutrophil function) was increased in unstimulated cells from MPD patients with and without *JAK2* mutation (Figure 2A). Stimulation of cells with fMLF markedly up-regulated CD11b cell surface expression in control neutrophils and neutrophils from MPD patients, but this fMLF-induced effect was not affected either by the disease or by *JAK2* mutation (Figure 2B).

P47phox phosphorylation on Ser345 is up-regulated in neutrophils isolated from patients with myeloproliferative disorders and is associated with the JAK2 V617F mutation

As p47phox phosphorylation on Ser345 is critical for NADPH oxidase hyperactivation and priming,[20] we analyzed this process in neutrophils from MPD patients. Interestingly, results show that Ser345 of p47phox was constitutively phosphorylated in neutrophils from MPD patients with the *JAK2* V617F mutation, contrary to neutrophils from healthy donors and MPD patients without *JAK2* mutation (Figures 3A-C). Ser345 is a MAPkinase phosphorylation site and can be phosphorylated by ERK1/2 and p38MAPkinase. Our results also show that the increase in Ser345 phosphorylation was associated with an increase in ERK1/2 phosphorylation of the upstream protein kinase (Figure 3A). Results shown in Figure 3D show again that p47phox and ERK1/2 are phosphorylated in neutrophils isolated from MPD patients with *JAK2* V617F mutation (JAK2 (V617F)+Buffer), as compared to neutrophils isolated from healthy donors (Control) and from MPD patients without *JAK2* mutation (JAK2-WT). Interestingly, incubation of neutrophils isolated from patients carrying the *JAK2* V617F mutation with the JAK2 inhibitor AG490 or the MEK1/2 inhibitor PD98059 resulted in the inhibition of the phosphorylation of p47phox on Ser345 and the phosphorylation of ERK1/2. Furthermore, GM-CSF, a cytokine known to activate JAK2 in neutrophils was able to enhance the phosphorylation of p47phox on Ser345 and the phosphorylation of ERK1/2 (Figure 3D). These results suggest that JAK2 controls ERK1/2 activation and that ERK1/2 are implicated in the phosphorylation of the NADPH oxidase subunit p47phox in neutrophils with the *JAK2* V617F mutation.

JAK2 controls GM-CSF-induced p47phox and ERK1/2 phosphorylation in neutrophils from healthy donors

To further demonstrate that JAK2 controls ERK1/2 activation, p47phox phosphorylation and upregulation of ROS production in normal neutrophils, we used GM-CSF, a cytokine known to activate JAK2, in neutrophils from healthy donors.[25-27] Neutrophils are terminally differentiated short-lived cells that are resistant to transfection. Therefore, an alternative strategy to study the role of specific enzymes in their functions is to use cell-permeant pharmacological inhibitors. To show the implication of JAK2 in GM-CSF-induced effects in neutrophils, we tested the effect of AG490, a selective JAK2 inhibitor.[28] As expect-

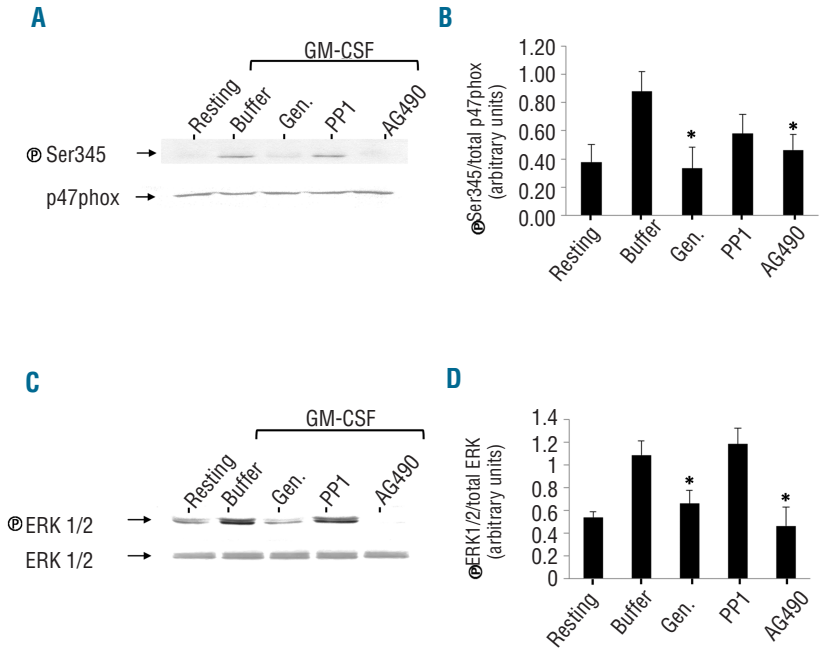

Figure 4. Effect of protein tyrosine kinase inhibitors on GM-CSF-induced phosphorylation of p47phox and ERK1/2. (A) Neutrophils were incubated with buffer, 100 μM genistein (Gen), 5 μM PP1 or 50 μM AG490 for 30 minutes, then with 12.5 ng/ml GM-CSF for 20 minutes. Total cell lysates (4 x 10^5 cells) were analyzed by SDS-PAGE and Western blot using the anti-phospho-Ser345 (pSer345) and anti-p47phox antibodies. (B) Scale bars represent means ± SEM (n=3, **P*<0.05). (C and D) Same as A and B except that Western blot was performed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Scale bars represent means ± SEM (n=3, **P*<0.05).

ed, GM-CSF induced p47phox phosphorylation on Ser345 (Figure 4A and B), and incubation of neutrophils with the broad-range tyrosine kinase inhibitor genistein or with the JAK2 inhibitor AG490 inhibited GM-CSF-induced p47phox phosphorylation on Ser345, whereas incubation with the src-tyrosine kinase inhibitor PP1 had no effect even at higher concentrations (*data not shown*). PP1 used in the same conditions is active, since it inhibited fMLF-induced ROS production by neutrophils (*data not shown*) as shown previously.[29] Labeling of p47phox with a specific antibody that recognizes both the phosphorylated and non-phosphorylated protein showed that the same amount of p47phox was present in each sample. As GM-CSF-induced p47phox phosphorylation on Ser345 is mediated by ERK1/2,[20] to determine whether JAK2 acts upstream of ERK1/2 phosphorylation, we tested the effects of tyrosine kinase inhibitors. Genistein and AG490 inhibited GM-CSF-induced ERK1/2 phosphorylation (Figure 4C and D), while PP1 had no effect, as for p47phox phosphorylation. ERK1/2 labeling with a specific antibody that recognizes both the phosphorylated and non-phosphorylated protein showed that the same amount of ERK1/2 was present in each sample.

To confirm the implication of JAK2 in GM-CSF-induced p47phox phosphorylation, we used lestaurtinib (CEP701), a more potent and selective inhibitor of JAK2.[30] AG490 and CEP701 inhibited GM-CSF-induced p47phox-Ser345 phosphorylation in human neutrophils in a concentration-dependent manner (Figure 5). At 100 μM of AG490 and 10 μM of CEP701, phosphorylation of p47phox was clearly inhibited. At the concentration used in this study, AG490 and CEP701 did not affect neutrophil viability, as shown by trypan blue exclusion test (*data not shown*).

The JAK2 inhibitor AG490 inhibits GM-CSF-induced NADPH oxidase hyperactivation and p47phox phosphorylation on Ser345

Results identify JAK2 as the upstream kinase of ERK1/2 activation pathway and, therefore, p47phox phosphorylation on Ser345. We then wanted to determine if the JAK2-ERK1/2-p47phox axis controls GM-CSF-induced priming of ROS production in neutrophils. We, therefore, tested the effects of the JAK2 inhibitor AG490 on GM-CSF-induced priming of ROS production. Interestingly, AG490 inhibited the priming effect of GM-CSF on fMLF-induced superoxide production in a concentration-dependent manner without affecting fMLF-induced stimulation (Figure 6A). The same AG490 concentrations inhibited p47phox phosphorylation on Ser345 (Figure 6B). CEP701 also inhibited GM-CSF-induced priming of fMLF-induced superoxide production (*data not shown*). These results clearly show that JAK2 is upstream ERK1/2 and p47phox phosphorylation on Ser345 and controls GM-CSF-induced NOX2 hyper-activation in neutrophils.

Discussion

In this study, we found that in neutrophils isolated from patients with myeloproliferative disorders associated with the *JAK2* V617F mutation, p47phox is phosphorylated on Ser345, and NADPH oxidase activity is up-regulated or primed, resulting in an increase in ROS production. In addition, when JAK2 is activated by GM-CSF, ERK1/2 phosphorylation and p47phox phosphorylation on Ser345 are both inhibited by JAK2 inhibitors. JAK2 inhibition resulted in a decrease in GM-CSF-induced priming of superoxide production by neutrophils.

Increased risk of thrombosis is associated with BCR-ABL1-negative chronic myeloproliferative disorders (MPD). Almost all patients with polycythemia vera and approximately half of those with essential thrombocythemia have this mutation. The *JAK2* V617F mutation results in a gain of function and induces constitutive tyrosine kinase activity. Neutrophil hyperactivation, reflected by elevated leukocyte alkaline phosphatase, plasma elas-

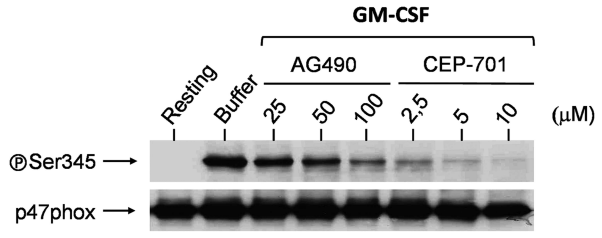

Figure 5. Effect of different concentrations of AG490 and CEP701 on p47phox phosphorylation on Ser345 in human neutrophils. Neutrophils were incubated with buffer, or with indicated AG490 or CEP701 concentrations for 30 minutes, then treated with 12.5 ng/mL GM-CSF for 20 minutes. Total cell lysates (4 x 10^5 cells) were analyzed by SDS-PAGE and Western blot using the anti-phospho-Ser345 (pSer345) and anti-p47phox antibodies (mean ± SEM, n=3).

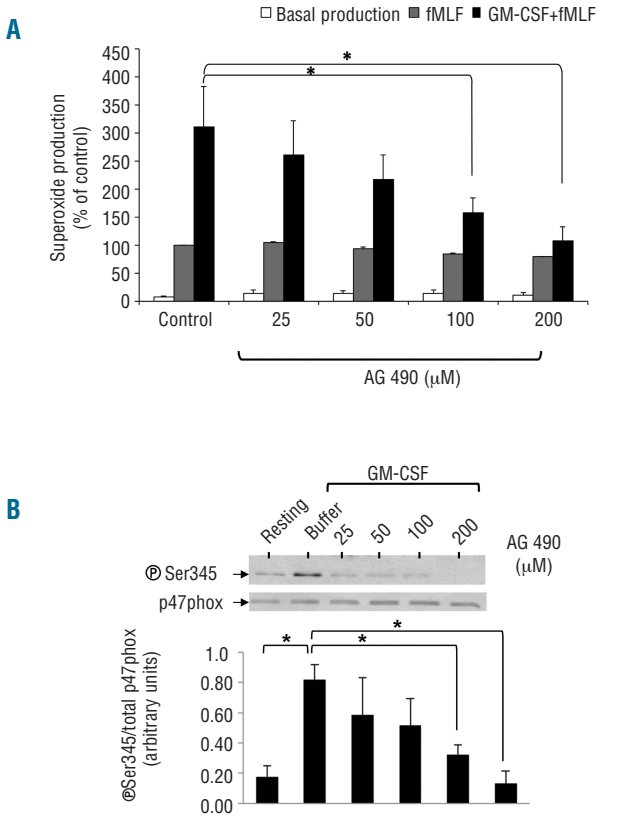

Figure 6. Effect of different concentrations of AG490 on neutrophil superoxide production and p47phox phosphorylation. (A) Neutrophils were incubated with or without AG490 for 30 min, then with 12.5 ng/ml GM-CSF for 20 min before stimulation with fMLF (10^{-7} M). Superoxide anion production was measured with the cytochrome c reduction assay at 550 nm as described in Methods. Control superoxide anion production (100%) was 3.9 nmoles/min/10^6 cells with fMLF alone (10^{-7} M). (B) Neutrophils were incubated with buffer or with indicated AG490 concentrations for 30 min, then with 12.5 ng/mL GM-CSF for 20 min. Total cell lysates (4 x 10^5 cells) were analyzed by SDS-PAGE and Western blot using the anti-phospho-Ser345 (pSer345) and anti-p47phox antibodies (mean±SEM, n = 5 *P<0.05).

tase and myeloperoxidase levels, has been implicated in the thrombophilia associated with MPD.[6-9] Here we show that neutrophils isolated from MPD patients with the *JAK2* V617F mutation are constitutively primed to produce ROS and that p47phox phosphorylation on Ser345 in these patients' neutrophils is up-regulated, confirming the upstream involvement of JAK2 in these processes. At the time of the present study, MPD patients with or without *JAK2* V617F mutation were treated with acetylsalicylic acid and/or hydroxyurea only. We did not notice any differences in ROS production due to the treatment.

In neutrophils, ROS can be generated by multiple sources such as the NADPH oxidase NOX2, mitochondria, xanthine oxidase and other enzymes. Our results show that DPI and apocynin almost completely blunted ROS production, suggesting that ROS production by MDP neutrophils is due to NOX2. It is clear that neutrophils are the major source of ROS in the blood; however, the contribution of ROS to the pathophysiology of MPD may have effects beyond these cells. Indeed, hydrogen peroxide (H2O2) can diffuse through cells and tissues, and can, therefore, affect other cells, such as endothelial cells. Excessive ROS levels have been shown to cause endothelial injury and to induce endothelial cells to release von Willebrand factor, a key factor in primary hemostasis.[10,11,13] Our findings show that aberrant JAK2 V617F activation is associated with excessive priming of neutrophil NADPH oxidase, leading to increased ROS production. This might play a role in the activation of endothelium and platelets, thus contributing to these patients' thrombophilic status.[7] Inhibition of the JAK2-ERK1/2-p47phox pathway might, therefore, serve as a novel therapeutic strategy.

To confirm the link between JAK2 and ROS production, we used GM-CSF, a cytokine known to activate JAK2 in neutrophils, and tested the effect of AG490 and CEP701, selective JAK2 inhibitors. The GM-CSF receptor is composed of two chains/subunits: a low-affinity alpha subunit (CD116) which is specific to GM-CSF, and a high-affinity beta chain (CD131) which is shared with IL-3 and IL-5.[31,32] GM-CSF binding to its receptor activates a number of signal transduction pathways in human neutrophils, including protein tyrosine kinases (PTK), PI3K[33,34] and the MAPK family ERK1/2.[35-39] GM-CSF binding to its receptor also induces and enhances the binding of the protein tyrosine kinase JAK2 and the src-tyrosine kinase lyn to the intracellular domain of the receptor.[25-27] These tyrosine kinases transduce the GM-CSF signal by phosphorylating other proteins such as STAT, PKB and PI3K. JAK2 is the only member of the JAK family to be activated by GM-CSF in neutrophils[25-27] and it is involved, directly or indirectly, in the tyrosine phosphorylation of p85, the regulatory subunit of PI3K.[25-27] We have previously shown that wortmannin, a PI3Kinase inhibitor, inhibits GM-CSF–induced p47phox phosphorylation,[40] supporting the idea that p47phox phosphorylation is controlled by the JAK2/PI3Kinase pathway. To study the role of JAK2 in p47phox phosphorylation, we used AG490 and CEP701 (also known as lestaurtinib) known to be selective JAK2 inhibitors.[28,30] AG490 inhibited phosphorylation of p47phox on Ser345 and phosphorylation of the upstream kinase ERK1/2, thus inhibiting NADPH oxidase hyperactivation and excessive ROS production by neutrophils. In order to verify if ROS contribute to GM-CSF-induced ERK1/2 phosphorylation and act via a positive loop for NOX2 activation, we tested the effect of DPI and apocynin on ERK1/2 phosphorylation. Surprisingly, DPI did not affect this process but apocynin did inhibit it (*results not shown*). As DPI and apocynin strongly inhibited neutrophil ROS production, the effect of apocynin on ERK1/2 phos-

phorylation is probably due to a non-related NADPH oxidase and ROS inhibitory effect.

We have recently shown that p47phox phosphorylation on Ser345 uncovers a binding site for the proline isomerase Pin1[21] and enhances p47phox phosphorylation by another protein kinase, and *per se* the NADPH oxidase activation induced by fMLF. Inhibition of JAK2 resulted in the inhibition of ERK1/2 activation and p47phox phosphorylation on Ser345 and could prevent Pin1 from binding to p47phox to enhance its phosphorylation and NADPH oxidase activation. All these data suggest that the "JAK2-PI3Kinase-ras-Raf-MEK-ERK1/2-p47phoxSer345-Pin1" axis controls GM-CSF-induced neutrophil priming. It is worthy of note that the JAK2 inhibitors specifically inhibited the priming effect of GM-CSF but did not alter superoxide production induced by fMLF alone, suggesting that JAK2 inhibitors might serve as an anti-inflammatory agent that preserves the bactericidal function of neutrophils.

In conclusion, this study shows that ROS production by neutrophils from MPD patients with *JAK2* V617F mutation is up-regulated and phosphorylation of p47phox on Ser345 is increased. The results also suggest that JAK2 controls GM-CSF-induced p47phox phosphorylation on Ser345, and neutrophil respiratory burst priming by GM-CSF. This JAK2/ERK/p47phox axis is over-activated in neutrophils from patients with the *JAK2* V617F mutation. Excessive ROS production controlled by this pathway in MPD disease could participate in endothelium injury and thrombosis reaction. Inhibition of this axis might inhibit the exaggerated neutrophil oxidative response but preserve the neutrophil beneficial function.

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