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Reactive Oxygen Species-Induced Activation of ERK and p38 MAPK Mediates PMA-Induced NETs Release from Human Neutrophils

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ABSTRACT

Neutrophils/polymorphonuclear leukocytes (PMNs), an important component of innate immune system, release extracellular traps (NETs) to eliminate invaded pathogens; however understanding of the role of signaling molecules/proteins need to be elucidated. In the present study role of p38 MAPK and extracellular signal regulated kinase (ERK) against phorbol 12-myristate 13-acetate (PMA) induced reactive oxygen species (ROS) generation and NETs formation has been investigated. Human neutrophils were treated with PMA to induce free radical generation and NETs release, which were monitored by NBT reduction and elastase/DNA release, respectively. PMA treatment led to the time dependent phosphorylation of p38 MAPK and ERK in PMNs. Pretreatment of PMNs with SB202190 or U0126 did not significantly reduce PMA induce free radical generation, but prevented NETs release. Pretreatment of PMNs with NADPH oxidase inhibitor (diphenyleneiodonium chloride) significantly reduced free radical generation, p38 MAPK and ERK phosphorylation as well as NETs release, suggesting that p38 MAPK and ERK activation was downstream to free radical generation. The present study thus demonstrates ROS dependent activation of ERK and p38 MAPK, which mediated PMA induced NETs release from human neutrophils. J. Cell. Biochem. 114: 532–540, 2013.

KEY WORDS: NEUTROPHIL EXTRACELLULAR TRAPS; NADPH-OXIDASE; SUPEROXIDE; ERK; p38 MAPK; FREE RADICALS

Neutrophils/polymorphonuclear leukocytes (PMNs), the first line of defence against the intruders, phagocytose and kill microbes by the combination of oxidative and non-oxidative mechanisms [Nathan, 2006]. Oxidative mechanism involves generation of reactive oxygen species (ROS) by NADPH oxidase complex while non-oxidative mechanism is dependent on the release of antimicrobial and microbicidal substances in phagolysosomes [Nathan, 2006]. Recently a novel mechanism of extracellular microbial killing by neutrophil extracellular traps (NETs) was reported [Brinkmann et al., 2004]. NETs are made up of chromatin embedded with nuclear and granular proteins [Brinkmann et al., 2004]. Yousefi et al. [2009] later reported that NETs, were made up of mitochondrial DNA [Yousefi et al., 2009]. NETs also contains cytoplasmic, cytoskeletal, peroxisomal, and glycolytic proteins [Urban et al., 2009].

Neutrophils do not exhibit classical apoptotic features prior to the release of NETs, it was therefore defined as NETosis [Steinberg and Grinstein, 2007], which was initially found dependent on the ROS [Fuchs et al., 2007], and was subsequently also found to be independent of ROS [Marcos et al., 2010]. The mechanism of NETs formation includes histone hypercitrullination by PAD 4 enzyme [Wang et al., 2009]. NETs had chromatin strands and anti-microbial proteins and proteases [Brinkmann et al., 2004], possibly to provide high concentration of antimicrobial proteins to prevent microbial spreading [Brinkmann et al., 2004]. It was recently reported from this lab that treatment of PMNs with NO donors released NETs involving NADPH oxidase and myeloperoxidase [Patel et al., 2010], which was mediated by the activation of p38 MAPK and extracellular signal regulated kinase (ERK) [Patel et al., 2008]. NO donor or phorbol 12-myristate 13-acetate (PMA) induced NETs

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contain mitochondrial as well as nuclear DNA and exhibit inflammatory potential [Keshari et al., 2012]. PMA, a strong stimulator of NETs formation [Brinkmann et al., 2004; Fuchs et al., 2007], activates classical and novel protein kinase Cs (PKCs) [Mellor and Parker, 1998; Toker, 1998]. PMA treatment leads to redistribution of PKC and phosphorylation of cytosolic p47 to activate NADPH oxidase in neutrophils, which plays a central role in host defense and inflammation [Dusi et al., 1996; Majumdar et al., 1991]. MAP kinases are central to the signal transduction pathways; however their role in NETs release was not known at the time, when study was initiated in this lab [Keshari et al., 2010]. To date, the three major MAPK families have been characterized: p38 MAPK, extracellular signal related kinase 1/2 (ERK1/2 or p44/42 MAPK), and stress-activated protein kinase (SAPK/JNK). Nonetheless, recent work has demonstrated that 2 of the MAPK families (ERK1/2 and p38 MAPK) are present in PMNs, and are activated within seconds by a variety of stimuli, including bacterial peptides, cytokines, lipopolysaccharides, and chemoattractants [Avdi et al., 1996; Nahas et al., 1996; Nick et al., 1996; Suchard et al., 1997]. p38 MAPK activation in macrophages and neutrophils induce respiratory burst activity, chemotaxis, granular exocytosis, adherence, apoptosis, and also mediates T-cell differentiation and apoptosis by regulating gamma interferon production [Ono and Han, 2000]. The aim of present study was to further assess the role of ERK and p38 MAPK in PMA induced free radicals and NETs release.

METHODS

ISOLATION OF HUMAN NEUTROPHILS

Blood from healthy volunteers was layered on Histopaque 1119 and centrifuged for 20 min at 800*g*. The lower interphase having granulocytes was collected and washed with RPMI 1640 medium, and was subsequently loaded on the discontinuous Percoll gradients as described earlier [Patel et al., 2010; Keshari et al., 2012]. Isolated PMNs were suspended in RPMI 1640 medium containing 0.5% FBS. The purity of the isolated PMNs was ascertained by CD15 staining using Flow cytometer (Becton Dickinson), which was never less than 95%. The study was approved by the institutional ethics committee and was conducted according to the Declaration of Helsinki.

NETs FORMATION

PMNs were seeded on 0.001% poly-L-lysine coated cover-slips, stimulated with PMA (20 nM), or H_2O_2 (50 μM) and incubated in CO_2 incubator at 37°C for 180 min. Effect of various interventions such as NADPH oxidase inhibitor [diphenyleneiodonium chloride (DPI, 10 μM)], p38 MAPK inhibitor [SB 202190, 20 μM)] and ERK inhibitor [U0126, 10 μM) was also monitored on NETs release by pre-incubating PMNs with DPI, SB202190, or U0126 at 37°C and then treated with PMA or H_2O_2 (50 μM) for 180 min. After fixation with 4% PFA and blocking, samples were stained overnight with 20 μg/ml of rabbit polyclonal elastase antibody (Calbiochem) and were visualized after treatment with the secondary antibody (1:200, chicken anti-rabbit AF 488 antibody, Molecular Probes) under a confocal microscope (Carl Zeiss LSM 510 META, Germany) and assessed for the incidence of NETs formation. DNA was stained with Hoechst 33258 (3 μg/ml) [Fuchs et al., 2007; Patel et al., 2010; Keshari et al., 2012]. The percentage of NETs was assessed by quantifying the number of NETs forming neutrophils out of the total number of neutrophils as observed under high-power fields at 400× magnification using a 40× objectives.

CYTOMETRIC BEAD ARRAY

PMNs were incubated with PMA or vehicle for 60 min. Cells were lysed in denaturation buffer and immediately boiled for 5 min at 100°C. Subsequent steps were performed according to the manufacturer's instruction (BD Biosciences). Briefly, lysate were mixed with capture beads and PE detection reagents. The samples were incubated for 4 h in dark, acquired on FACS Aria (BD Biosciences) and subsequently analyzed by using FCAP software [Morgan et al., 2004].

QUANTIFICATION OF DNA RELEASE

Neutrophils (1×10^6 cells) were seeded on 0.001% poly-L-lysine coated 24 well plates and subsequently stimulated with PMA (20 nM) for 180 min. Plates were centrifuged for 5 min at 500*g*, supernatant was collected, and NETs bound DNA was released by incubating sample with medium containing 500 mU/ml of micrococcal nuclease (MNase; Worthington Biochemical Corp.). 5 mM EDTA was added to stop the nuclease activity, samples were centrifuged, supernatant was collected and stored at 4°C. Total DNA was isolated from unstimulated neutrophils (1×10^6 cells) with DNazol supplemented with 1% polyacryl carrier (Molecular Research Center) according to the manufacturer's instructions and solubilized in TE buffer. DNA was stained by adding 5 μM of Sytox green (Invitrogen), a non-cell-permeable DNA-binding dye and fluorescence was recorded at λ_{ex} 488 nm and λ_{em} of 520 nm by using spectrofluorimeter (Varian Eclipse fluorescence spectrophotometer, Netherlands). NET bound DNA release has been reported as % NET bound DNA with the PMNs (1×10^6) total DNA content [Brinkmann et al., 2004; Fuchs et al., 2007].

WESTERN BLOTTING

Neutrophils (2×10^6) were stimulated with PMA (20 nm) or H_2O_2 (50 μM) at 37°C for 15-180 min, centrifuged at 3,000 rpm for 15 min, pellets were lysed in 150 μl of Laemmli sample buffer containing protease and phosphatase (PTP) inhibitor cocktail and boiled for 5 min at 100°C. Samples were centrifuged at 13,000*g* for 15 min. Supernatant (30 μl) was run on the 10% SDS polyacrylamide gel, and transferred to PVDF membranes. Membranes were blocked with blocking solution (TBST containing 5% BSA) for 60 min at room temperature. Membrane was washed with TBST (TBS containing 0.1% Tween-20) and incubated with phospho-p38, total p38, phospho-ERK1/2, total ERK1/2 (1:1,000, cell signalling technology) antibody overnight, after washing membranes were incubated with HRP-conjugated secondary antibody for 120 min. The immune complexes were visualized by enhanced chemiluminescence using ECL kit (Amersham Biosciences, Uppsala, Sweden).

NITROBLUE TETRAZOLIUM (NBT) REDUCTION ASSAY

Neutrophils (1×10^6) were pre-incubated with vehicle, DPI, SB 202190, or U0126 and then 10 μM NBT was added. Subsequently PMA (20 nM) was added to induce superoxide generation for 30 min at 37°C. Cells were washed twice with PBS, lysed by adding 120 μl

2 M KOH and 140 μl DMSO. Contents were properly mixed for 10 min by vortexing at room temperature and absorbance was read at 620 nm (BioTek) [Choi et al., 2006].

STATISTICAL ANALYSIS

Results have been expressed as mean ± SEM of three to five independent experiments. Multiple comparisons were made by one-way ANOVA followed by Newman–Keuls post analysis test. Results were considered significant at $P < 0.05$.

RESULTS

PMA INDUCED NETs FORMATION WAS INHIBITED BY NADPH OXIDASE, ERK, AND p38 MAPK INHIBITOR

PMA stimulated NETs formation was visualized by elastase and DNA staining. Resting neutrophils (Fig. 1A) stained with elastase antibody and Hoechst demonstrated punctated pattern of elastase distribution and multilobed nuclei. PMNs treated with PMA for 180 min however, lost their morphology and released NETs, which were evident by the colocalization of elastase and DNA (Figs. 1B and 3A). DPI (10 μM), a NADPH oxidase inhibitor, prevented PMA induced NETs formation (Figs. 1D and 3A). Pretreatment of PMNs with p38 MAPK (SB202190, 20 μM) or ERK1/2 (U0126, 10 μM) inhibitor prevented the PMA induced NETs formation (Fig. 2B, D). DPI, SB 202190, or U0126 per se had no effect on the spontaneous NETs formation (Figs. 1C and 2A, C). We also used exogenous hydrogen peroxide (H_2O_2) to induced NETs formation. Neutrophil incubated with H_2O_2 induced NETs formation, which was reduced by the p38 MAPK and ERK inhibitor suggesting that H_2O_2 induced NETs formation was dependent on ERK and p38 MAP kinases (Fig. 3A). PMA induced NETs formation was significantly more than that of H_2O_2 (Fig. 3A).

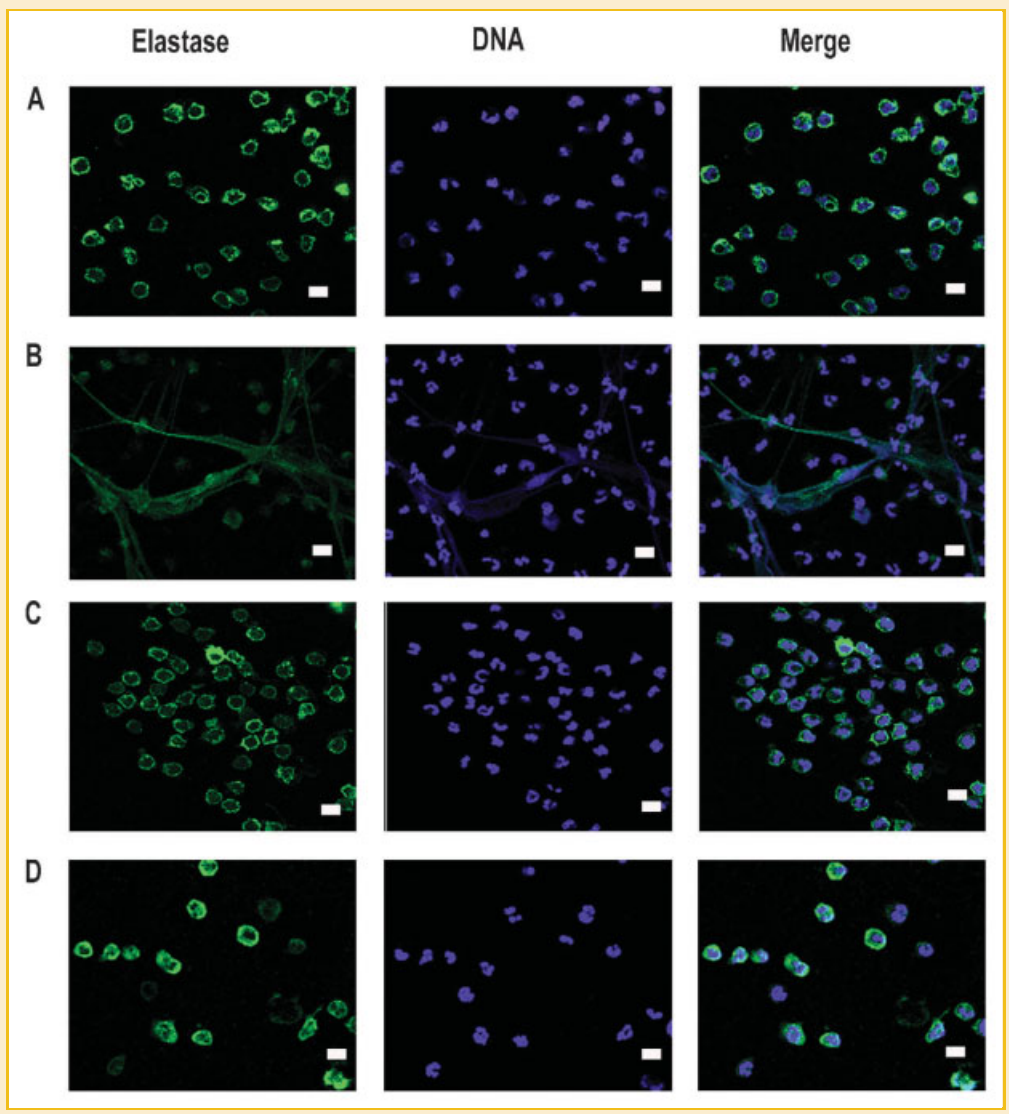

Fig. 1. PMA induced NETs formation. A: Resting neutrophils stained with elastase antibody conjugated with AF 488 (green) and Hoechst 33258 (blue) showing multilobed nuclei and punctate elastase. B: PMNs incubated with PMA (20 nM) for 180 min released NETs. C: PMNs pre incubated with DPI, (D) PMN incubated with DPI and PMA for 180 min exhibit inhibition of NETs formation (bar A–D 10 μm).

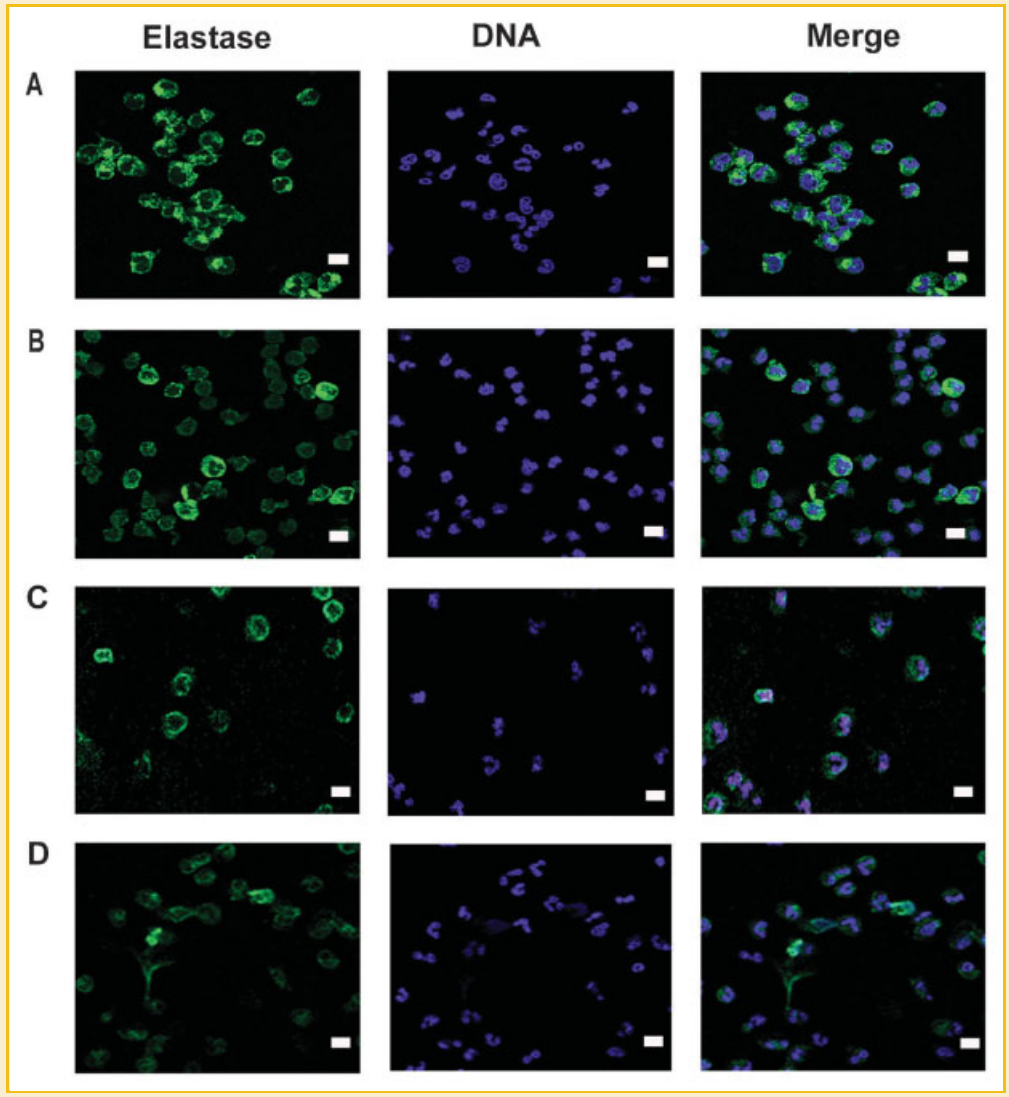

Fig. 2. SB 202190 or U0126 inhibited PMA induced NETs formation. A: PMNs pre incubated with SB202190, (B) SB 202190 and PMA for 180 min prevented NETs formation. C: PMNs pre incubated with U0126, (D) U0126 and PMA for 180 min inhibited NETs formation (bar A–D 10 μm).

DPI, SB 202190, AND U0126 INHIBITED THE PMA INDUCED DNA RELEASE

Effect of DPI, SB 202190, or U0126 was also monitored on DNA release in the medium following PMA treatment for 180 min. Incubation of PMNs with PMA induced significant release of DNA as compared to control, which was reduced by DPI, SB202190, or U0126 pre-treated PMNs confirming the role of free radicals, p38, and ERK MAPK, respectively, in PMA induced NETs release (Fig. 3B).

DPI INHIBITED THE PMA INDUCED SUPEROXIDE GENERATION

Superoxide generation was determined by NBT assay. Incubation of PMNs with PMA significantly enhanced generation of superoxide radicals, which was prevented by the pre-treatment with NADPH oxidase inhibitor (DPI). However SB 202190 or U0126 failed to reduce PMA induced superoxide generation (Fig. 3C).

PMA AND H_2O_2 TRIGGERS PHOSPHORYLATION OF ERK1/2 AND p38 MAP KINASE

p38 MAPK are involved in multiple processes such as growth, differentiation, oxidative stress and inflammation, and has been reported to be induced by PMA. The ERK1/2 cascade is also activated through receptor-mediated signaling stimuli including growth factors and phorbol esters and leading to cell proliferation, differentiation, and survival. The time dependent phosphorylation of ERK and p38 MAPK was monitored following PMA treatment. Significant increase in phosphorylation of both p38 MAPK and ERK1/2 following PMA treatment was evident (Fig. 3D–F). p38 MAPK phosphorylation was significantly and consistently increased in a time dependent manner at 60, 120, 180 min, while ERK 1/2 phosphorylation was maximal at 30 and 60 min, and subsequently ERK 1/2 phosphorylation was reduced at 120 and 180 min as compared to 30 and 60 min, however it was still significantly more

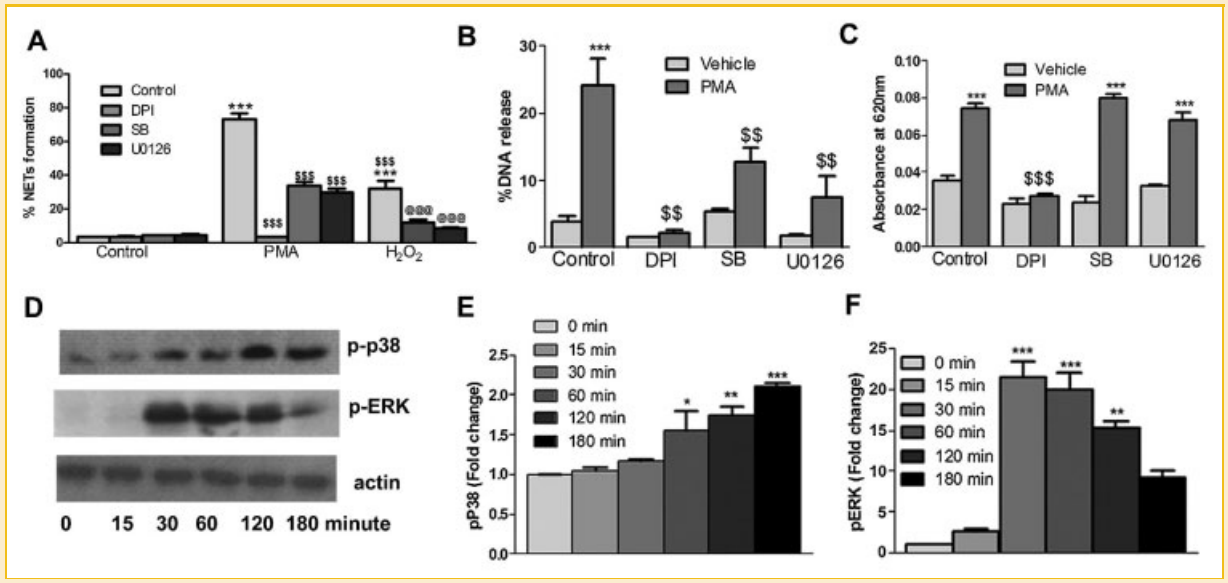

Fig. 3. PMA induced NETs formation, DNA release, superoxide generation, ERK, and p38 MAPK phosphorylation. A: PMN incubated with PMA for 180 min released NETs which was inhibited by DPI, SB 202190 or U0126 ($^{***}P < 0.001$ vs. control; $^{\$\$\$}P < 0.001$ vs. PMA treated PMNs, $^{@@@}P < 0.001$ vs. H_2O_2 treated PMNs). H_2O_2 induced NETs were inhibited by SB 202190 or U0126 (data presented as % release, are mean count ± SEM of five transect from three individual experiments. B: Quantification of NET bound DNA following MNase (500 mU/ml) treatment ($^{***}P < 0.001$ vs. control; $^{\$\$}P < 0.01$ vs. PMA treated PMNs). C: PMA induced superoxide production was inhibited by DPI, however SB 202190 and U0126 was unable to reduce PMA induced superoxide production ($^{***}P < 0.001$ vs. control; $^{\$\$\$}P < 0.001$ vs. PMA treated PMNs). D: PMNs were incubated with PMA (20 nM) at 37°C for the various time intervals and lysed. Lysates were electroblotted and stained with various antibodies as described in methods section. Data are representative of experiments repeated at least three times with similar results. E: Densitometry of phospho-p38 MAPK and (F) phospho-ERK MAPK ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control).

than control at 120 min (Fig. 3D–F). Since NETs formation was dependent on the free radicals, we further observed the effect of DPI on PMA induced phosphorylation of p38 and ERK1/2 MAPK by Western blotting and cytometric bead array. PMNs incubated with PMA for 60 min exhibited significant phosphorylation of p38 MAPK and ERK 1/2 (Fig. 4A, B). DPI inhibited PMA induced phosphorylation of p38 MAPK and ERK 1/2, which was confirmed by both Western blotting (Fig. 4C, D) and cytometric bead array (Fig. 4A, B). PMNs pretreated with ERK inhibitor U0126 (10 μM) inhibited the PMA induced phosphorylation of ERK (Fig. 4D). Cross regulation of p38 MAPK and ERK was studied by using their inhibitors. Pretreatment of ERK inhibitor had no effect on PMA induced p38 MAPK phosphorylation, while p38 MAPK inhibitor reduced the PMA induced ERK phosphorylation, suggesting the regulation of ERK activation by p38 MAPK (Fig. 5A–C). H_2O_2 treatment also induced the p38 MAPK and ERK phosphorylation. H_2O_2 induced ERK phosphorylation was reduced by the U0126 (Fig. 5D, F). p38 MAPK inhibitor had no effect on H_2O_2 induced ERK phosphorylation and similarly ERK inhibitor had no effect on p38 MAPK phosphorylation. DPI had no effect on H_2O_2 induced ERK and p38 MAPK phosphorylation (Fig. 5D–F).

DISCUSSION

Neutrophil extracellular traps are web like structures, which are expelled by these cells to extracellularly entrap and kill the microorganisms. NADPH oxidase [Fuchs et al., 2007] and myeloperoxidase [Papayannopoulos et al., 2010; Patel et al., 2010] activation and histone hypercitrullination [Wang et al., 2009] have been associated with NETs formation. CXCR2 mediated NETs formation was, however, independent of NADPH oxidase activation and was mediated through Src family kinases [Marcos et al., 2010]. NADPH oxidase is activated by a variety of agents, including fMLP and the PKC activator, PMA [Wientjes and Segal, 1995]. In the present study, we have demonstrated that PMA induced NETs formation was mediated by superoxide production, which subsequently phosphorylated p38 MAPK and ERK to promote NETs release [Keshari et al., 2010]. PMA induced NETs formation was visualized by immunolabeling and DNA release, which was significantly reduced by SB 202190, U0126, and DPI, demonstrating the role of p38 MAPK, ERK, and NADPH oxidase, respectively (Figs. 1, 2, and 3B). H_2O_2 induced NETs formation was mediated by the ERK and p38 MAPK activation (Fig. 3A). Recently Lim et al. [2011] reported that NETs formation was impaired in neutrophil lacking Rac 2 (a cytosolic subunit of NADPH oxidase and important in ROS generation) and this effect was rescued by the exogenous treatment of H_2O_2.

Since NETs formation was induced in the immobilized cells, we also assessed superoxide generation by using NBT reduction method in the immobilized PMNs [Choi et al., 2006]. PMA induced superoxide generation was significantly inhibited by NADPH oxidase inhibitor (DPI); however SB 202190 or U0126, did not affect superoxide generation (Fig. 3C), suggesting that p38 MAPK and ERK were downstream to the superoxide formation. It has been reported that PMA induced superoxide production was also not

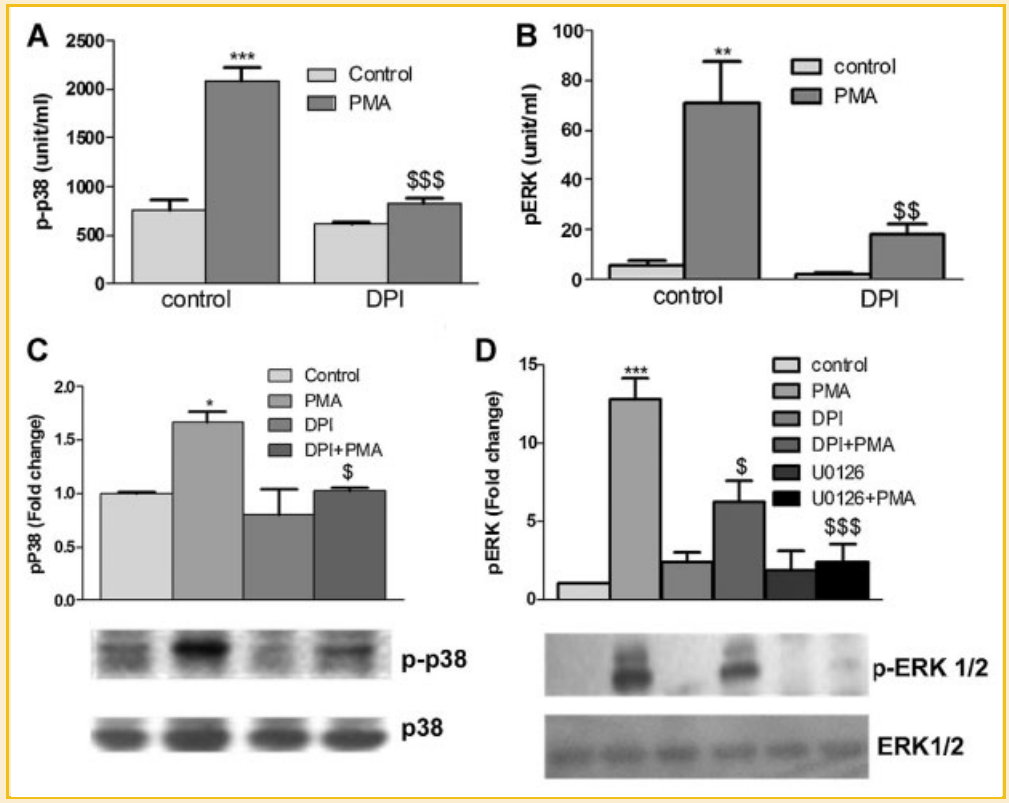

Fig. 4. DPI inhibited the PMA induced ERK and p38 MAPK phosphorylation. A: PMNs incubated with PMA for 60 min and then p38 MAPK and (B) ERK phosphorylation was assessed by bead array as described in the method section ($^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs. PMA treated PMNs). C: Phosphorylation of p38 MAPK was investigated in presence of DPI ($^{*}P < 0.05$ vs. control; $^{\$}P < 0.05$ vs. PMA treated PMNs). D: Phosphorylation of ERK1/2 MAPK was determined in presence of DPI and ERK inhibitor U0126 ($^{***}P < 0.001$ vs. control; $^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$ vs. PMA treated PMNs).

inhibited by SB 203580, a p38 MAPK inhibitor [Detmers et al., 1998; Partrick et al., 2000]. p38 MAPK however, plays an important role in the neutrophil degranulation [Mocsai et al., 2000; Lacy, 2006]. Contrary to the present study Hakkim et al., [2010] observed that U0126 inhibited free radical generation as well as NETs release. The concentration of U0126, used by them was 100 μM, which might have non-specific effects [Kawamata et al., 2003], moreover luminol was used by them to assess free radical generation, which detects both extracellular and intracellular ROS ($O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, HOCl, $^{\bullet}NO$, $ONOO^-$) [Freitas et al., 2009]. In addition they did not mention whether the experiments to assess the free radical generation were performed in immobilized or suspended cells, which differ in the time course of free radical release [Yoshida et al., 1999]. In the present study we used NBT, to detect intracellular superoxide radicals in the immobilized cells [Freitas et al., 2009]. The observed discrepancy could thus be due to any of the above mentioned reasons. We observed phosphorylation of p38 MAPK [Keshari et al., 2010] and ERK 1/2 in PMA induced NETs formation in a time dependent manner. Significant increase in ERK 1/2 phosphorylation was found at 30 min, and after 120 min it exhibited a tendency of decrease (Fig. 3D, F). p38 MAPK phosphorylation was seen later at 60 min, and it remained sustained up to 180 min (Fig. 3D, E). Our data is in agreement to the previous report demonstrating ERK activation at early time point which was followed by activation of p38 MAPK [Junttila et al., 2008]. p38 MAPK negatively regulates the ERK activity. Direct interaction between p38 MAPK and ERK1/2 has been proposed as a mechanism to inhibit ERK1/2 phosphorylation and activity [Zhang et al., 2001]. Effect of p38 MAPK and ERK inhibitor was therefore assessed on PMA induced ERK and p38 MAPK phosphorylation. ERK inhibitor failed to affect p38 MAPK phosphorylation, while p38 MAPK inhibitor inhibited the ERK phosphorylation, suggesting the regulation of ERK activation by p38 MAPK (Fig. 5). Moreover p38 MAPK and ERK phosphorylation was inhibited by NADPH oxidase inhibitor, confirming the role of free radicals in p38 MAPK and ERK 1/2 activation. Present study thus demonstrates that ERK was phosphorylated earlier than p38 MAPK and also that free radicals generation was upstream to the kinase activation. NADPH oxidase-derived ROS-mediated activation of ERK1/2 has been reported during Entamoeba-induced neutrophil apoptosis [Sim et al., 2005], PMA and hydrogen peroxide induced apoptosis [Takei et al., 1996; Tsan, 1980; Tsan and Denison, 1980], which was independent of caspase activation. Moreover caspase activation was also not observed in NETs formation [Fuchs et al., 2007]. ERK also prevents cell death mediated by death receptors (Fas, TNFR, and TRAIL) and caspase 8 activation [Tran et al., 2001]. Activated p38 MAPK and ERK are thus important for neutrophils survival by preventing caspase activation [Alvarado-Kristensson et al., 2004]. It has been shown that PTPs are inactivated

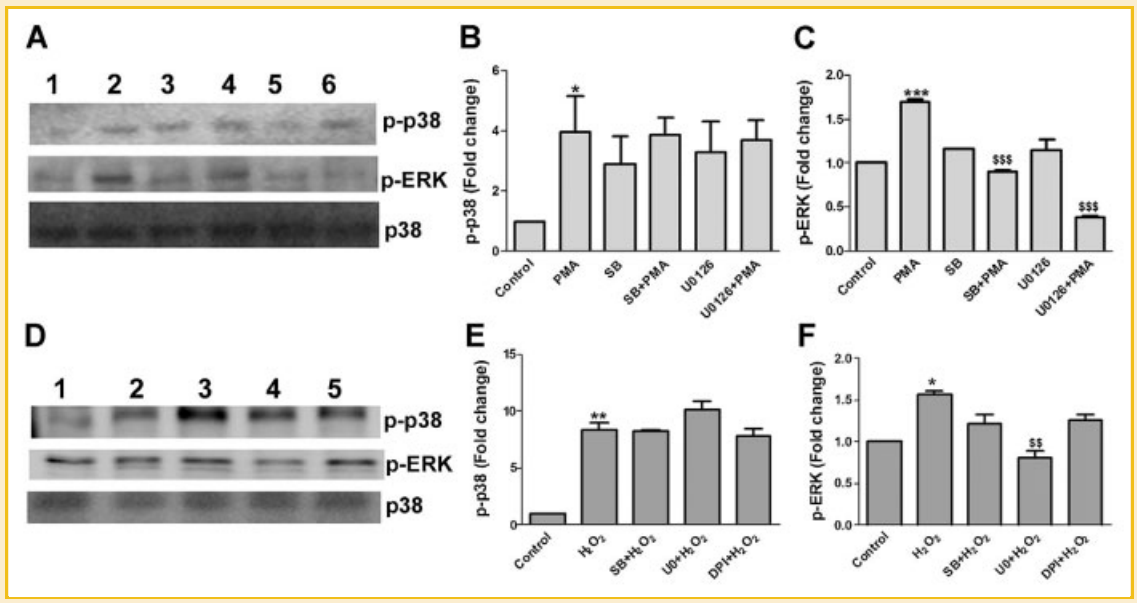

Fig. 5. Effect of SB 202190 and U0126 on ERK and p38 MAPK phosphorylation. A: ERK and p38 MAPK phosphorylation in presence of SB 202190 and U0126 (Lane 1-control, Lane 2-PMA, Lane 3-SB 202190, Lane 4-SB + PMA, Lane 5-U0126, Lane 6-U0126 + PMA). B: Densitometric analysis of p38 MAPK phosphorylation of (A; $^{*}P < 0.05$ vs. control). C: Densitometric analysis of ERK phosphorylation of (A; $^{***}P < 0.001$ vs. control; $^{\$\$\$}P < 0.001$ vs. PMA treated PMNs). D: ERK and p38 MAPK phosphorylation in presence of SB 202190 and U0126 (Lane 1-control, Lane 2-H_2O_2, Lane 3-SB 202190 + H_2O_2, Lane 4-U0126 + H_2O_2, Lane 5-DPI + H_2O_2. E: Densitometric analysis of p38 MAPK phosphorylation of (D; $^{**}P < 0.01$ vs. control). F: Densitometric analysis of ERK phosphorylation of (D; $^{*}P < 0.05$ vs. control; $^{\$\$}P < 0.01$ vs. PMA treated PMNs). Data are representative of experiments repeated at least three times with similar results.

by the ROS (H_2O_2), which regulate the activation of distinct MAPK pathways [Lee and Esselman, 2002]. It is therefore important to assess the role of phophatases and kinases in the sustained phosphorylation of p38 MAPK and ERK during NETs generation. H_2O_2 treatment also induced the ERK and p38 MAPK phosphorylation which was reduced by their respective inhibitors. In contrast to PMA, p38 MAPK inhibitor had no effect on H_2O_2 induced ERK phosphorylation and ERK inhibitor had no effect on H_2O_2 induced p38 MAPK phosphorylation (Fig. 5D–F). Houliston et al. [2001] reported the agonist specific cross talk between ERK and p38 MAPK regulate PGI_2 synthesis following stimulation of HUVEC cells with thrombin and IL-1α. SB-203580 pre-treatment enhanced IL-1 α-induced ERK phosphorylation but reduced thrombin-stimulated ERK phosphorylation.

This study demonstrates for the first time that although PMA and H_2O_2 induced NET formation may involve free radicals and activation of p38 MAPK and ERK pathway, their regulation may differ since in case of PMA, activation of ERK was dependent on p38 MAPK activation however in case of H_2O_2 this was not the case. More importantly this is the first report demonstrating time dependent phosphorylation and activation of p38 MAPK during NET formation. Present results also suggest that PMA is a more potent agonist than H_2O_2 when it comes to NET formation. We used a most commonly used H_2O_2 concentration [Lim et al., 2011]. This observation indicates that different pools of free radicals are generated by H_2O_2 and PMA treatment which may show different potency to NET formation. Recent reports suggests the singlet oxygen and hypochlorous acid induced NETs formation [Nishinaka et al., 2012; Palmer et al., 2012]. It will be interesting to identify specific free radical species/enzymes contributing to maximum NET release.

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