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Journal of Cellular Biochemistry 113:899–910 (2012)

Journal of Cellular Biochemistry

Restraint of Spreading-Dependent Activation of Polymorphonuclear Leukocyte NADPH Oxidase in an Acidified Environment

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ABSTRACT

Elucidation of the mechanisms by which environmental pH affects or regulates the functions of polymorphonuclear leukocytes (PMNs) is important because severe acidification of the microenvironment often prevails at sites of inflammation where they act in host defense. In the present study, we investigated the effect of an acidic environment on spreading-dependent activation of O_2^--producing NADPH oxidase in PMNs. We found that PMNs underwent spreading spontaneously over type I collagen and plastic surfaces at both neutral and acidic pH, although spreading over fibrinogen surfaces, for which cellular stimulation with H_2O_2 is required, was inhibited by acidic pH. At acidic pH, however, PMNs were unable to undergo spreading-dependent production of O_2^-. Pharmacological experiments showed that p38 mitogen-activated protein kinase (MAPK) was involved in the signaling pathways mediating the spreading-dependent activation of NADPH oxidase, and that its spreading-dependent phosphorylation of Thr-180 and Tyr-182, a hallmark of activation, was impaired at acidic pH. Furthermore, the inhibition by acidic pH of O_2^- production as well as p38 MAPK phosphorylation subsequent to spreading induction was reversible; environmental neutralization and acidification after induction of spreading at acidic and neutral pH, respectively, up- and down-regulated the two phenomena. Acidic pH did not affect the O_2^- production activity of NADPH oxidase pre-activated by phorbol 12-myristate 13-acetate (PMA). These results suggest that, in PMNs, the p38 MAPK-mediated signaling pathway functions as a pH-sensing regulator of spreading-dependent NADPH oxidase activation. J. Cell. Biochem. 113: 899–910, 2012.

KEY WORDS: POLYMORPHONUCLEAR LEUKOCYTE; NADPH OXIDASE; p38 MAPK; SPREADING; ACIDIC ENVIRONMENT

Polymorphonuclear leukocytes (PMNs) are among the first leukocytes to reach an inflammatory site and play a critical role in host defense against infection. NADPH oxidase-mediated production of the superoxide anion (O_2^-) is well known to be an important microbicidal function of PMNs that accumulate at sites of infection [Bokoch and Knaus, 2003]. The active form of NADPH oxidase is a membrane-associated multi-component enzyme complex made up of membrane-bound $gp91^{phox}$ and $p22^{phox}$, which together form flavocytochrome b_{558}, and cytosolic components including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac 2, which translocate to the membrane upon cellular activation [Kim and Dinauer, 2001; Bokoch and Knaus, 2003]. Activation of PMN NADPH oxidase involves interaction of $p47^{phox}$ and $p22^{phox}$ [Bokoch and Knaus, 2003], an event for which extensive phosphorylation of serine residues within the C-terminal region of $p47^{phox}$ by protein kinase C (PKC) is required [Ago et al., 1999]. The importance of NADPH oxidase in host defense has been clearly confirmed by studies of genetic defects that affect microbial killing (chronic granulomatous disease) [Roos et al., 1996]. On the other hand, inappropriate or excessive activation of NADPH oxidase in PMNs is implicated in tissue-damaging inflammatory reactions. Accordingly, tightly regulated activation of PMNs is important for successful host defense.

In physiological situations, integrin-dependent firm adhesion to biological surfaces is an absolute requisite for activation of NADPH oxidase in PMNs [Nathan et al., 1989; Detmers et al., 1998; Williams and Solomkin, 1999]. It is well established that, upon ligand binding, integrins provide powerful intracellular signals (referred to as outside-in signaling) that regulate many cellular responses in leukocytes as well as other cells [Lowell and Berton, 1999]. Importantly, in PMNs, β_2 integrins have an indispensable role in NADPH oxidase activation resulting from adhesion mediated by not only β_2 but also β_1 and β_3 integrins [Nathan et al., 1989; Williams and Solomkin, 1999; Chen et al., 2003]. Extensive studies of signaling pathways (outside-in signaling) from ligand binding of β_2 integrins to activation of NADPH oxidase have demonstrated the

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Received 3 October 2011; Accepted 14 October 2011 • DOI 10.1002/jcb.23416 • © 2011 Wiley Periodicals, Inc.
Published online 22 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

involvement of several proteins such as Src-family protein tyrosine kinases [Lowell and Berton, 1999], plastins (actin-crosslinking proteins) [Chen et al., 2003], Syk kinases [Lowell and Berton, 1999; Chen et al., 2003], Vav family of Rho GTPase guanine nucleotide exchange factors (GEFs) and phospholipase C-γ2 (PLC-γ2) [Graham et al., 2007], PKC [Kilpatrick et al., 2010], p38 mitogen-activated protein kinase (p38 MAPK) [Lynch et al., 1999; Kilpatrick et al., 2010; Lavigne et al., 2006], and PML-retinoic acid receptor α-regulated adaptor molecule 1 (PRAM-1) [Clemens et al., 2004]. However, precise details of the pathways coupling adhesion with NADPH oxidase activation still remain to be elucidated, because such studies are complicated by the need to differentiate between the pathways that enhance integrin-ligand binding (referred to as inside-out signaling) and those that mediate cellular activation by integrin-ligand binding (outside-in signaling) [Lynch et al., 1999]. Moreover, information about the state of cell morphology is important for clarifying the mechanisms underlying integrin-dependent activation of NADPH oxidase, because, in addition to activation of each of the signaling molecules mediating specific integrin-induced signaling pathways, morphological flattening of the PMN cell body following cell attachment is thought to contribute to effective operation of these pathways by bringing these signaling effectors into close proximity [Williams and Solomkin, 1999]. For example, PMNs deficient in Src family kinases (*hck/fgr*), plastin, or PRAM-1 are unable to produce O_2^- even though the latter two mutants undergo normal spreading, indicating that plastin and PRAM-1 are required components of the signaling pathways responsible for integrin-dependent activation of NADPH oxidase while Src kinases mediate induction of spreading [Lowell and Berton, 1999; Chen et al., 2003; Clemens et al., 2004].

In most in vitro studies, the mechanism underlying the regulation of PMN functions has been investigated by experiments in which PMNs are cultured under neutral pH conditions. PMN functions are largely affected by changes in environmental pH (and thus intracellular pH). Generally, PMN functions including cell spreading and NADPH oxidase activation are known to be impaired by cytoplasmic acidification, whereas they are up-regulated by cytoplasmic alkalization [Nasmith and Grinstein, 1986; Araki et al., 1991; Yuo et al., 1993; Demaurex et al., 1996; Suzuki and Namiki, 2007]. Severe acidification of the microenvironment often prevails at the sites of inflammation where they act in host defense [Sawyer et al., 1991]. For this reason, careful in vitro studies taking into account environmental pH are necessary for a precise understanding of the functional regulation of PMNs in vivo. In our laboratory, we have investigated the effects of environmental pH on PMN spreading, and searched for conditions under which PMNs can spread over fibrinogen deposits at acidic pH, in the expectation that PMNs may employ some specific functional regulation system adapted to the acidified milieu [Suzuki and Namiki, 2007, 2009; Suzuki et al., 2008].

In the present study, we assessed the effect of an acidic environment on O_2^- production by PMNs after induction of spreading over a variety of surfaces including fibrinogen, type I collagen, and plastic. We found that PMNs are able to spread well over type I collagen or plastic surfaces even under acidic conditions, although they are unable to undergo subsequent spreading-dependent O_2^- production. Pharmacological experiments indicated that p38 MAPK is involved in the signaling pathways that mediate spreading-dependent activation of NADPH oxidase, and that p38 MAPK activation induced by cell spreading is impaired at acidic pH. We also demonstrated that the p38 MAPK-mediated post-spreading signaling pathways act as a reversible pH sensor responsible for on/off switching of NADPH oxidase activation.

MATERIALS AND METHODS

MATERIALS

Phenylmethanesulfonylfluoride (PMSF), human fibrinogen, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), catalase, cytochrome *c* (oxidized form), and cytochalasin B were purchased from Sigma (St. Louis, MO). 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), 2′-amino-3′-methoxyflavone (PD98059), 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide (Gö6983), and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazole[3,4-d]pyrimidine (PP2) were from Calbiochem (San Diego, CA). Methyl cellulose 25cP, superoxide dismutase (SOD), dimethyl sulfoxide (DMSO), H_2O_2, 3,3′-diaminobenzidine tetrahydrochloride dehydrate (DAB) and phorbol 12-myristate 13-acetate (PMA) were from Wako Pure Chemical (Tokyo, Japan). Plain culture plate (polystyrene, 96-well or 100 mm dish) was from Asahi Glass (Tokyo, Japan). Biocoat Collagen I Cellware 96-well plate was from BD Biosciences (Bedford, MA). Ficoll-Paque Plus was from GE Healthcare Bio-Science AB (Uppsala, Sweden). EDTA-free Complete Mini protease inhibitor cocktail (tablets) was from Roche Diagnostics (Mannheim, Germany). Fresh porcine blood was obtained from Tokyo Shibaura Zoki (Tokyo, Japan). Rabbit anti-p38 MAPK antibody (NT) was from AnaSpec (San Jose, CA). Rabbit anti-phospho p38 MAPK antibody (pThr180/Tyr182) was from AbD Serotec (Oxford, UK). HRP-conjugated goat anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the highest purity grade.

PREPARATION OF IMMOBILIZED FIBRINOGEN SURFACES

A culture plate with immobilized fibrinogen was prepared by incubation with 1 mg/ml fibrinogen for 2 h at 4°C (non-denaturing) or with 10 μg/ml fibrinogen for 24 h at 37°C (denaturing) followed by washing.

CELL SPREADING

PMNs were isolated routinely from porcine peripheral blood, and PMN spreading was assessed morphologically as described previously [Suzuki et al., 2008]. Briefly, PMNs suspended in pH-adjusted HEPES-NaCl buffer (10 mM HEPES, 140 mM NaCl, 5 mM glucose) were pretreated with or without inhibitors at the indicated concentrations for 60 min at 37°C, then plated onto immobilized protein surfaces or uncoated plastic surfaces and incubated for 30 min at 37°C in the presence or absence of divalent cations (0.6 mM Mg^{2+} and 2 mM Ca^{2+}) and/or 400 μM H_2O_2. Photomicrographs were taken and the cells depicted on them were counted. Those that were phase dark, and enlarged with irregular shapes, were considered spread, and their percentages were calculated.

DETECTION OF O_2^- RELEASED BY PMNs

O_2^- generated by activated PMNs was detected as the rate of cytochrome *c* reduction [Suzuki et al., 2010] with modifications. Immediately after photomicrographs had been taken for assessment of cell spreading, cytocrome *c* solution (50 μM cytochrome *c*, 100 μg/ml catalase) was added, and incubation was conducted for the indicated time periods. Cytochrome *c* reduced by O_2^- was detected by measuring the SOD-inhibitable increase in absorbance at 550 nm (A_{550}). Because non-specific reduction of cytochrome *c* was not detectable when assays using live PMNs had been performed in the presence of SOD (Figs. 1–5), the data were not shown.

CELL-FREE NADPH OXIDASE ASSAY

Enzyme activity of plasma membrane-associated NADPH oxidase was assessed under cell-free conditions after isolation of the PMN plasma membrane fraction [Suzuki et al., 2008]. First, PMNs in suspension (at pH 7.2 or 6.0) were stimulated with 1 μg/ml PMA for 10 min at 37°C, and then disrupted by sonication after washing and re-suspension in disruption buffer (HEPES-NaCl buffer containing 1 mM PMSF and protease-inhibitor cocktail). PMN homogenates were centrifuged at 500*g* for 10 min to precipitate nuclei and debris. The post-nuclear supernatant was further centrifuged at 279,000*g* for 40 min to precipitate the plasma membrane fraction. The plasma membrane pellet was re-suspended in ice-cold disruption buffer at pH 7.2 or 6.0, and used immediately for the cell-free NADPH oxidase assay. The PMN plasma membrane fraction (4.5×10^7 cell equivalents/ml) was mixed with 50 μM cytochrome *c*, 100 μg/ml catalase and 2 mM NaN_3, and the A_{550} was monitored for 1 min at room temperature. Four hundred micromolar β-NADPH was then added to initiate the enzyme reaction of NADPH oxidase, and monitoring of A_{550} was continued for up to 4 min. The reaction was allowed to proceed in both the absence and presence of 500 U/ml

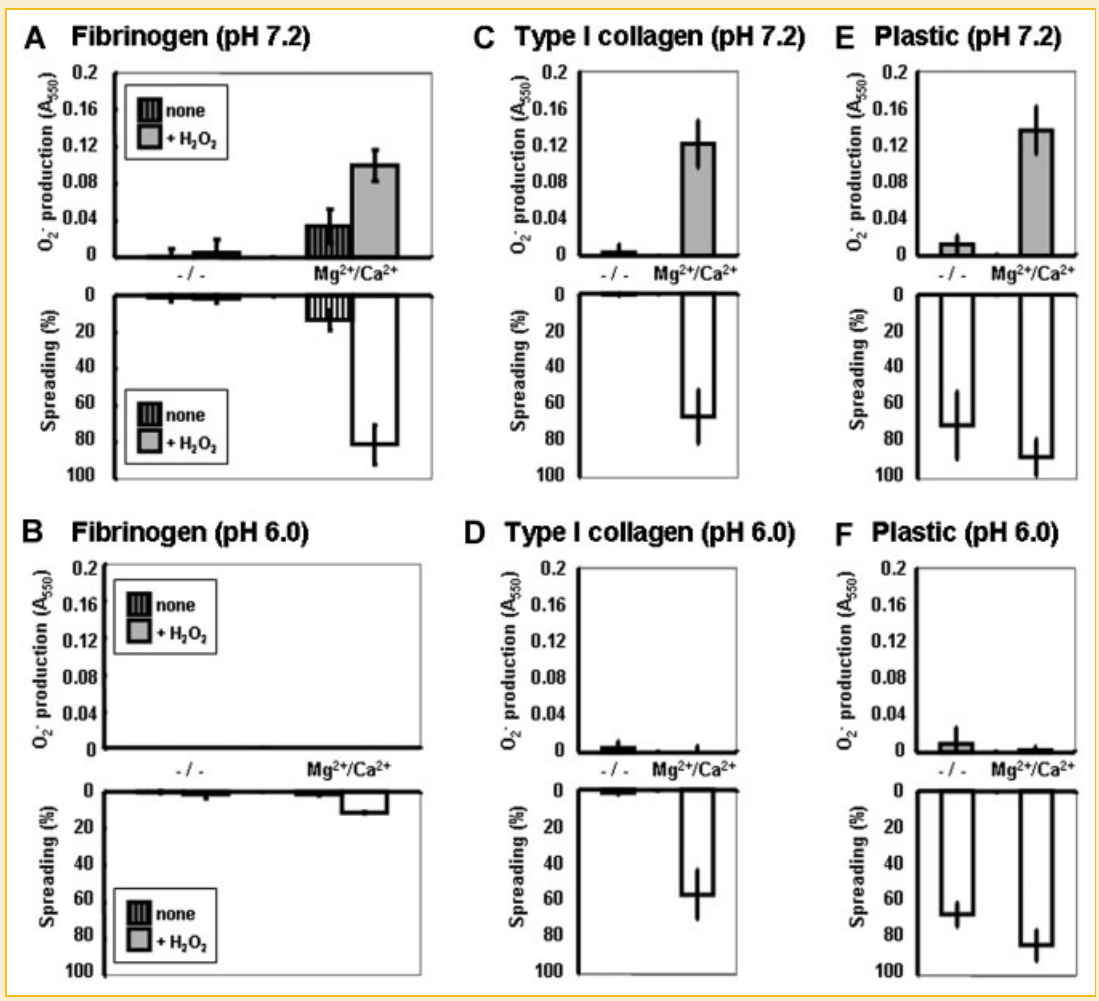

Fig. 1. Spreading and O_2^- production by PMNs at pH 7.2 and 6.0. PMNs were plated onto surfaces of immobilized fibrinogen (A and B), immobilized type I collagen (C and D), and plastic (E and F), and incubated for 30 min at 37°C in the presence or absence of divalent cations at pH 7.2 (A, C, and E) or 6.0 (B, D, and F). In (A) and (B), PMNs were stimulated with or without 400 μM H_2O_2 when they had been plated onto fibrinogen surfaces. Immediately after photomicrographs had been taken for assessment of cell spreading (lower graphs), cytochrome *c* solution was added, and incubation was continued for a further 90 min. Cytochrome *c* reduced by O_2^- was then quantified by measuring the absorbance at 550 nm, and then subtracting the absorbance at the incubation time period of 0 min (upper graphs). Data are expressed as the mean ± S.D. of at least three independent experiments.

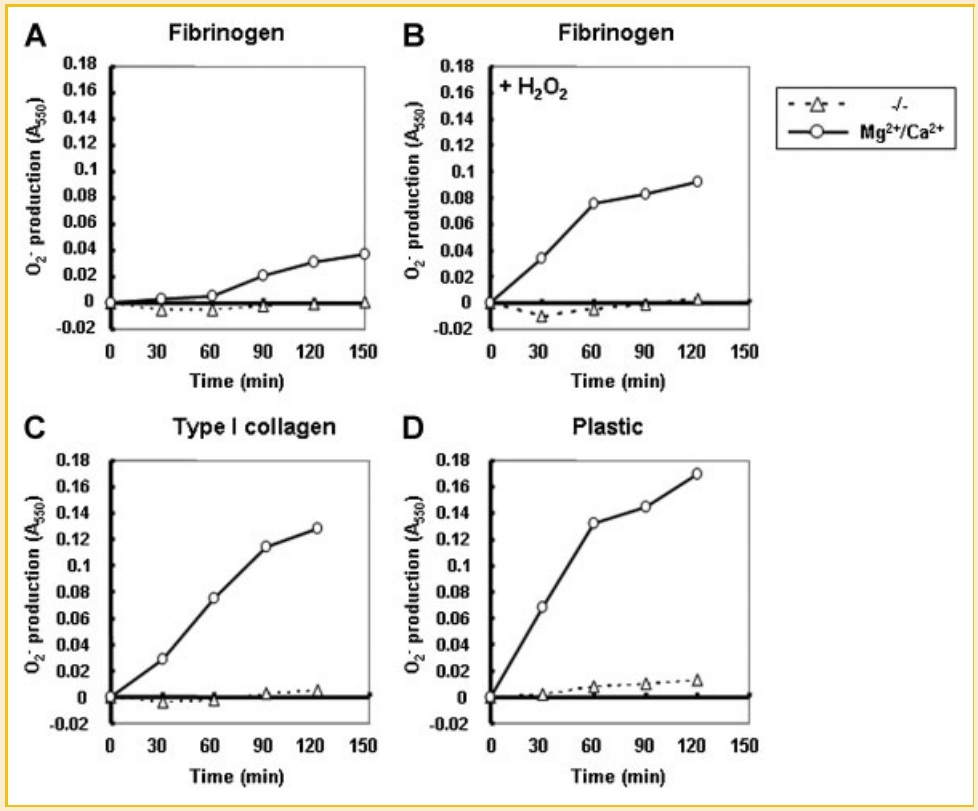

Fig. 2. Time course of O_2^- production by PMNs spread over surfaces of immobilized proteins or plastic at pH 7.2. O_2^- production was monitored by cytochrome *c* reduction assay after PMNs had been induced to spread over surfaces of fibrinogen (A and B), type I collagen (C), and plastic (D), as described in Figure 1. In (B), PMNs were plated together with 400 μM H_2O_2 onto fibrinogen surfaces. Data shown are representative of two independent experiments.

SOD in order to distinguish the O_2^--dependent reduction of cytochrome *c* (NADPH oxidase activity) from non-specific reduction of the molecule.

ANALYSIS OF p38 MAK ACTIVATION IN ADHERENT PMNs

Activation of p38 MAPK was assessed by detection of the dual phosphorylation of Thr-180 and Tyr-182 [Raingeaud et al., 1995; Bouaouina et al., 2004]. PMNs (1.2×10^7 cells) were plated onto a 100-mm-diameter plastic dish, and then spreading was induced by incubation for 30 min at 37°C in the presence or absence of divalent cations at the indicated pH. After removal of the culture supernatant, the cells were lysed by addition of 500 μl of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM $MgSO_4Y \cdot 7H_2O$, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 1 mM PMSF, and protease inhibitor cocktail) followed by scraping. After the cell lysate had been pre-cleared by centrifugation at 18,000*g* for 5 min, cellular proteins were precipitated by addition of 10% trichloroacetic acid and mixing, followed by two washes with ice-cold ethanol and centrifugation. The protein pellet was dissolved in reduced SDS sample buffer and analyzed by SDS–PAGE using 10% polyacrylamide gels, and electrophoretically transferred to Immobilon™-P membranes (Millipore, Bedford, MA). After membrane blocking with 5% non-fat dried milk, phosphorylated p38 MAPK was probed using a rabbit antibody raised against p38 MAPK phosphorylated at Thr-180/Tyr-182. Total p38 MAPK in each sample was probed using a phosphorylation state-independent anti-p38 MAPK antibody. For detection of the protein bands, the DAB color reaction was used after incubation with an HRP-conjugated secondary antibody [Suzuki and Namiki, 2007].

RESULTS

COMPARISON OF SPREADING-DEPENDENT O_2^- PRODUCTION BY PMNs AT NEUTRAL pH WITH THAT AT ACIDIC pH

We attempted to examine whether PMNs could produce O_2^- when they had undergone spreading in an acidified environment. We first tested the effect of pH on O_2^- production by PMNs after induction of spreading over immobilized fibrinogen surfaces, having the β_2 integrin-specific epitopes [Ugarova et al., 1998; Hu et al., 2001; Mosesson, 2005]. At pH 7.2 (Fig. 1A), PMNs underwent marked spreading over immobilized fibrinogen surfaces and in a divalent cation-dependent manner when the cells were stimulated with H_2O_2. As expected, spreading-dependent reduction of cytochrome *c* (O_2^- production) was observed when cytochrome *c* had been added 30 min after plating of PMNs and incubated for a further 90 min at this pH (Fig. 1A). At pH 6.0, PMNs stimulated by H_2O_2 showed only weak spreading over fibrinogen surfaces in the presence of divalent cations, and O_2^- production was not observed (Fig. 1B). We next performed experiments using immobilized type I collagen surfaces,

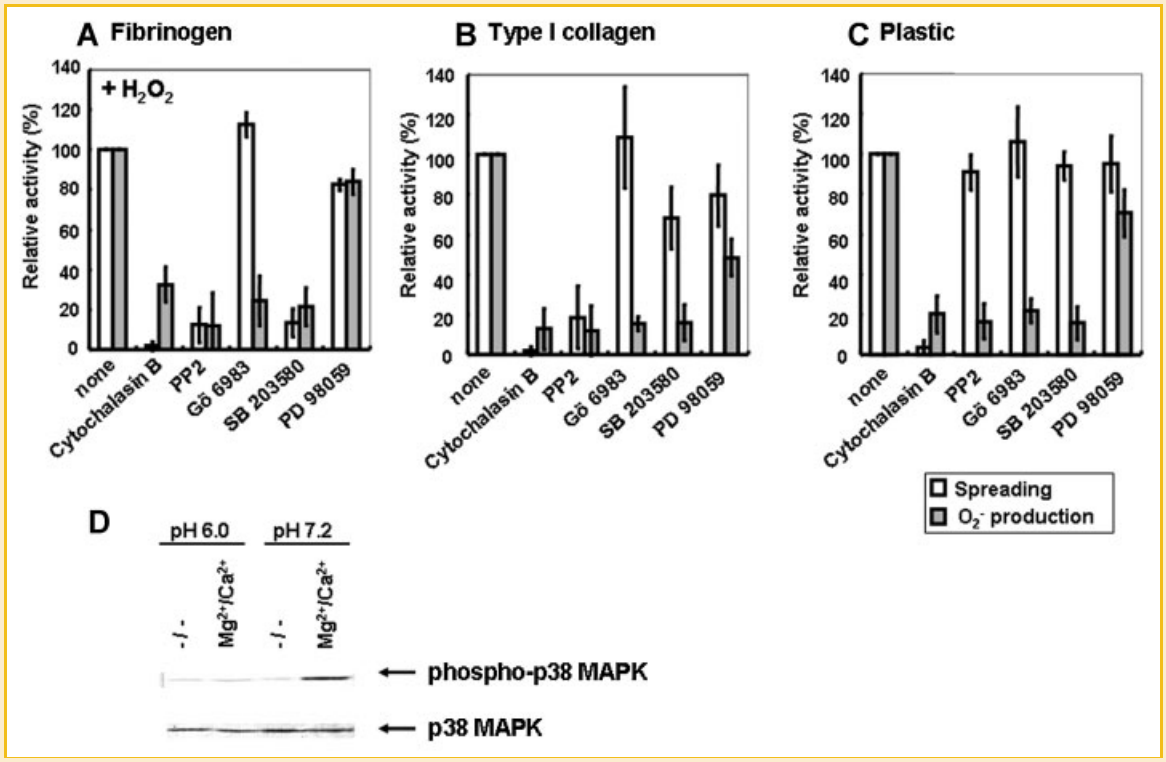

Fig. 3. (A–C) Effect of pharmacological inhibitors on spreading and subsequent O_2^- production by PMNs at pH 7.2. PMNs were pre-treated for 60 min at 37°C with 5 μg/ml cytochalasin B, 30 μM PP2, 10 μM Gö6983, 10 μM SB203580, and 50 μM PD98059, respectively, before being plated onto surfaces of fibrinogen (A), type I collagen (B), and plastic (C). PMN spreading and O_2^- production were induced and assessed in the presence of divalent cations, as described in Figure 1. In (A), PMNs were plated together with 400 μM H_2O_2 onto fibrinogen surfaces. Data are expressed as the mean relative spreading (clear bars) and relative O_2^- production (solid bars) ± S.D. for at least three independent experiments, in which the spreading and O_2^- production in the absence of inhibitors were considered as 100%. (D) Phosphorylation of p38 MAPK in PMNs spread over plastic surfaces at pH 7.2 and 6.0. PMNs were induced to spread over plastic surfaces by incubation for 30 min at 37°C in the presence or absence of divalent cations, and at pH 7.2 or 6.0. Cell lysates (6×10^6 cell equivalents) were analyzed by immunoblotting using anti-phosphorylated p38 MAPK (Thr-180/Tyr-182) antibody. Total content of p38 MAPK was determined using phosphorylation state-independent anti-p38 MAPK antibody. Data shown are representative of two independent experiments.

which have also been reported to induce β_2 integrin-mediated spreading and NADPH oxidase activation of PMNs [Garnotel et al., 1995; Sud'ina et al., 2001]. When PMNs were plated onto type I collagen surfaces, marked and divalent cation-dependent spreading was induced even at pH 6.0 (Fig. 1D), as well as at pH 7.2 (Fig. 1C). On the other hand, however, spreading-dependent O_2^- production was observed only at pH 7.2, but not at pH 6.0 (Fig. 1C,D). We also assessed spreading and O_2^- production by PMNs plated onto plastic surfaces. Adhesion and spreading of PMNs over plastic surfaces is known to be an integrin-independent, non-specific adhesion process [Wright et al., 1989; Kori et al., 2009]. As shown in Figure 1E,F, PMNs underwent marked spreading both in the presence and absence of divalent cations, and irrespective of pH. Nevertheless, spreading-dependent O_2^- production occurred only at pH 7.2 (Fig. 1E), and not at pH 6.0 (Fig. 1F). Importantly, PMNs spread over plastic surfaces under divalent cation-free conditions could not produce O_2^- even at pH 7.2 (Fig. 1E).

Representative data from time course analyses of PMN O_2^- production after induction of spreading are shown in Figure 2. Ferricytochrome *c* was added 30 min after plating of PMNs onto each of the surfaces, and this time point was considered as "0 min", since 30 min was required for sufficient induction of PMN spreading and, especially in experiments using plastic surfaces, proteins such as cytochrome *c* and catalase affected the efficiency of spreading induction when they were added to the wells together with PMNs (data not shown). PMN spreading over all three surfaces was stable throughout the incubation period (data not shown).

EFFECT OF PHARMACOLOGICAL INHIBITORS ON SPREADING-DEPENDENT O_2^- PRODUCTION

To study the signaling pathways involved in spreading-dependent O_2^- production by PMNs, we investigated the effects of pharmacological inhibitors on PMN spreading and subsequent production of O_2^- at pH 7.2. PMNs are terminally differentiated, short-lived cells, that are resistant to transfection. Therefore, cell-permeant inhibitors can be used as an alternative tool for studying the role of specific enzymes in PMN function. PMNs were pretreated with each inhibitor for 60 min at 37°C before plating onto surfaces. The results of experiments using fibrinogen surfaces, type I collagen surfaces, and plastic surfaces are shown in Figure 3A,B,C, respectively.

We previously reported that cytochalasin B, an inhibitor of F-actin assembly, inhibited the spreading of PMNs over fibrinogen surfaces [Suzuki and Namiki, 2007]. Cytochalasin B has also been

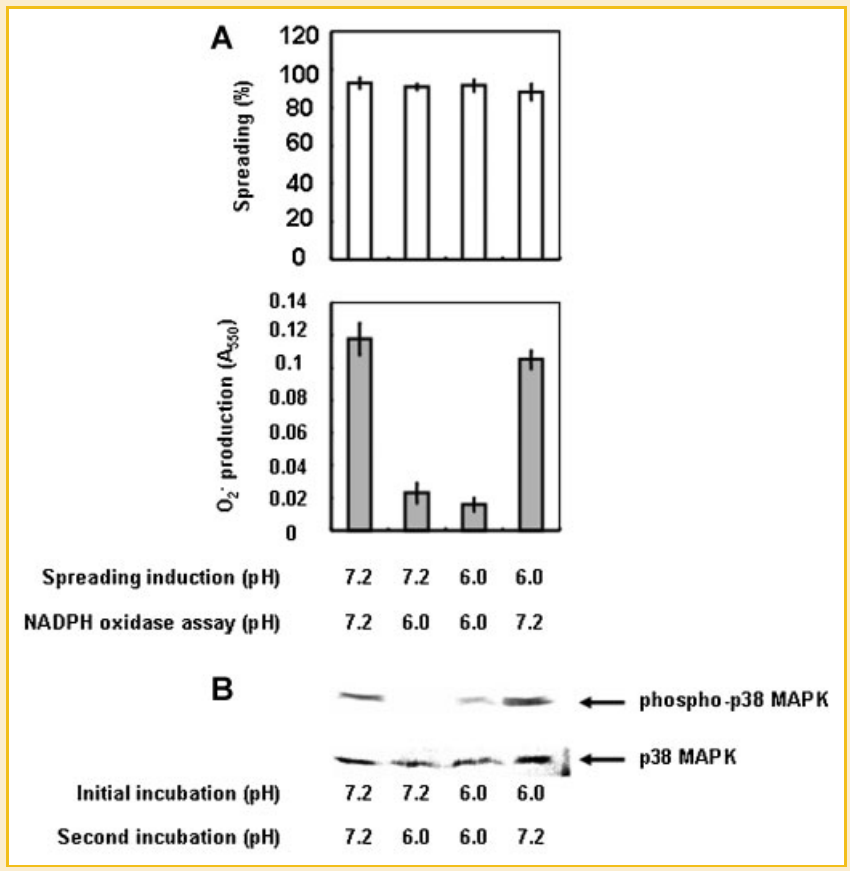

Fig. 4. Reversible inhibition of spreading-dependent O_2^- production and p38 MAPK phosphorylation in PMNs. (A) PMNs in suspension (pH 7.2 or 6.0) were plated together with divalent cations onto plastic surfaces, and spreading was induced by incubation for 30 min at 37°C. Immediately after photomicrographs had been taken for assessment of cell spreading (upper graphs), the medium was carefully aspirated and exchanged for cytochrome c solution at the indicated pH, and incubation was continued for a further 90 min. O_2^- production was assessed as reduction of cytochrome c, as described in Figure 1 (lower graphs). Data are expressed as the mean ± S.D. of four independent experiments. (B) PMNs were plated together with divalent cations onto plastic surfaces and spreading was induced as described in (A) (Initial incubation). Thereafter the medium was carefully aspirated and exchanged for fresh medium at the indicated pH, and the cells were further incubated in the presence of divalent cations for 30 min at 37°C (Second incubation). Cell lysates were then prepared and phosphorylation of p38 MAPK was analyzed as described in Figure 3. Data shown are representative of two independent experiments.

reported to inhibit H_2O_2 release by PMNs adhered to immobilized anti-β_2 integrin mAb surfaces [Berton et al., 1992]. In agreement with the previous reports, cytochalasin B markedly inhibited both spreading and O_2^- production, irrespective of the surfaces onto which PMNs had been plated.

The central role of Src family protein tyrosine kinases in β_2 integrin outside-in signaling in PMNs is also well established [Lowell and Berton, 1999; Lynch et al., 1999; Bouaouina et al., 2004]. Expectedly, PP2, a specific Src kinase inhibitor, strongly inhibited spreading as well as O_2^- production of PMNs plated onto surfaces coated with integrin-specific ligands including fibrinogen and type I collagen (Fig. 3A,B, respectively). On the other hand, while PP2 had no effect on integrin-independent spreading of PMNs over plastic surfaces, it still inhibited the subsequent production of O_2^- by PMNs (Fig. 3C).

PKC reportedly becomes activated following cell adhesion to, and spreading over extracellular matrix surfaces [DeMali et al., 2003]. In PMNs, PKC activation has also been implicated in spreading-dependent production of O_2^-, while induction of spreading has been reported to be PKC-independent [Lynch et al., 1999; Graham et al., 2007; Suzuki and Namiki, 2009]. As shown in Figure 3A–C, Gö6983, a specific but broad-spectrum PKC inhibitor, had no inhibitory effect on induction of PMN spreading, irrespective of the surfaces onto which they had been plated, whereas the agent strongly inhibited subsequent O_2^- production by the spread PMNs.

The MAPKs including p38 MAPK and ERK1/2 become activated following integrin stimulation, and are thought to be necessary for activation of effector functions in PMNs [Detmers et al., 1998; Lowell and Berton, 1999; Bouaouina et al., 2004; Clemens et al., 2004; Lavigne et al., 2006; Pluskota et al., 2008]. We therefore examined whether pharmacological inhibitors of these kinases affected spreading and subsequent O_2^- production by PMNs. When PMNs that had been pretreated with SB203580, a specific inhibitor of p38 MAPK [Cuenda et al., 1995], were plated onto fibrinogen surfaces and stimulated with H_2O_2, both spreading and O_2^- production were markedly inhibited (Fig. 3A). Interestingly, however, the PMN spreading over the other two surfaces was largely resistant to pretreatment with SB203580 (Fig. 3B,C).

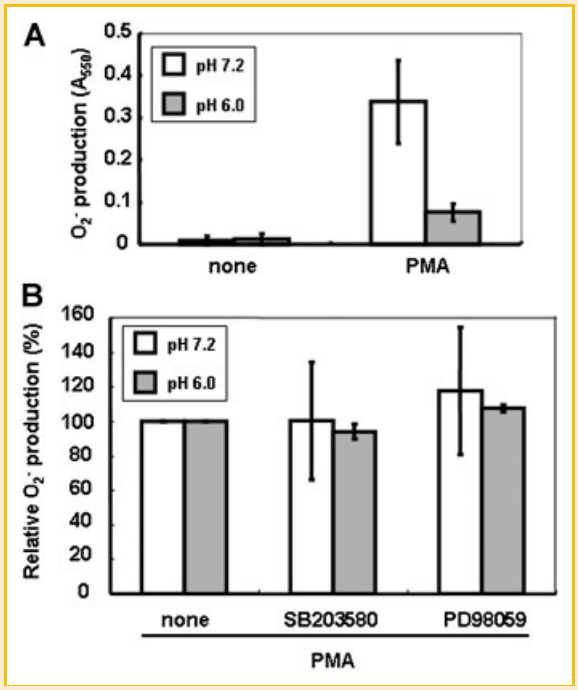

Fig. 5. Spreading-independent O_2^- production by PMNs stimulated with PMA. (A) PMNs in suspension were stimulated with 1 μg/ml PMA for 30 min at 37°C in the presence of cytochrome *c* and catalase. Cytochrome *c* reduced by O_2^- was quantified as described in Figure 1. Data are expressed as the mean ± S.D. of four independent experiments. (B) PMNs were pre-treated for 60 min at 37°C with 10 μM SB203580 or 50 μM PD98059 before stimulation with PMA. Data are expressed as the mean relative O_2^- production ± S.D. of three independent experiments, in which the O_2^- production in the absence of inhibitors were considered as 100%.

Whereas the spreading of PMNs over fibrinogen surfaces upon stimulation with H_2O_2 following pretreatment with SB203580 was 13.3 ± 6.9%, relative to spreading in the absence of the inhibitor considered as 100% (Fig. 3A), the spontaneous spreading of SB203580-treated PMNs over type I collagen surfaces and plastic surfaces was 68.4 ± 15.3% (Fig. 3B) and 94.3 ± 6.9% (Fig. 3C), respectively. Nevertheless, SB203580 strongly inhibited the spreading-dependent production of O_2^- by PMNs irrespective of the surfaces onto which they had been plated (Fig. 3A–C). In comparison with the inhibitory effects of SB203580 on spreading and O_2^- production by PMNs, those of PD98059, an inhibitor of ERK1/2, were relatively small (Fig. 3A–C).

ACTIVATION OF p38 MAPK AFTER INDUCTION OF INTEGRIN-MEDIATED SPREADING AT pH 7.2, BUT NOT AT pH 6.0

The activation of p38 MAPK is mediated by dual phosphorylation on Thr-180 and Tyr-182 [Raingeaud et al., 1995]. We therefore addressed the issue of whether environmental pH affected the phosphorylation of p38 MAPK in PMNs spread over plastic surfaces. We used only plastic surfaces for the experiments, because spreading of PMNs over the surfaces was independent of pH and divalent cations (Fig. 1E,F), and we were able to examine the effect of these factors on phosphorylation of p38 MAPK. In contrast, PMNs spread over fibrinogen surfaces only at pH 7.2 (Fig. 1A,B), and it would be difficult to comprehend the data of experiments using fibrinogen surfaces because cellular stimulation with H_2O_2 might modulate the activity of p38 MAPK [Bouaouina et al., 2004]. The analyses using type I collagen surfaces were also unable to be performed in the present study because we could not obtain commercially available 100-mm-diameter dish pre-coated with the same grade type I collagen. PMNs were incubated on plastic surfaces in the presence or absence of divalent cations, at a pH of 7.2 or 6.0. Cell lysates were then prepared and phosphorylation of p38 MAPK was evaluated by immunoblotting using anti-phosphorylated p38 MAPK (Thr-180/Tyr-182) antibody. As shown in Figure 3D, phosphorylation of p38 MAPK was markedly promoted in a divalent cation-dependent manner at pH 7.2. However, the divalent cation-dependent phosphorylation of p38 MAPK was abrogated at pH 6.0.

REVERSIBLE REGULATION OF POST-SPREADING p38 MAPK AND NADPH OXIDASE ACTIVATION BY pH

We examined whether the process of pH-dependent activation of NADPH oxidase in PMNs after induction of spreading was reversible. As shown in Figure 4A, similar level of PMN spreading was observed at pH 7.2 and 6.0 when PMNs were plated over plastic surfaces and incubated for 30 min in the presence of divalent cations. At this time point, the medium was carefully exchanged for cytochrome *c* solution with a pH 7.2 or 6.0, and incubation was continued for a further 90 min. Interestingly, environmental acidification resulted in marked suppression of O_2^- production even when PMNs had been induced to spread at pH 7.2. In contrast, environmental neutralization after induction of PMN spreading at pH 6.0 led to marked production of O_2^-. The effect of medium (pH) exchanges on the phosphorylation status of p38 MAPK was also assessed; cell lysates were prepared at the incubation time point of 30 min after medium (pH) exchange, and phosphorylation of p38 MAPK was evaluated by immunoblotting as described above. In PMNs initially incubated for 30 min at pH 7.2 (spreading induction) and then additionally incubated for 30 min after medium exchange without pH modification, marked phosphorylation of p38 MAPK was observed (Fig. 4B). However, environmental acidification after induction of PMN spreading at pH 7.2 markedly lowered the level of p38 MAPK phosphorylation (Fig. 4B). Moreover, in comparison to the spreading induction at pH 6.0 followed by medium exchange without pH modification, environmental neutralization after spreading induction at pH 6.0 markedly promoted the phosphorylation of p38 MAPK (Fig. 4B).

ACIDIC ENVIRONMENT SUPPRESSES SPREADING- AND p38 MAPK-INDEPENDENT ACTIVATION BUT NOT THE ENZYME ACTIVITY OF NADPH OXIDASE IN PMNs STIMULATED WITH PMA

PMA, a direct agonist of PKC, has been reported to induce β_2 integrin- and p38 MAPK-independent activation of NADPH oxidase in PMNs [Nathan et al., 1989; Nick et al., 1997; Zu et al., 1998], despite the fact that PMA induces phosphorylation of p38 MAPK and the kinase is reportedly able to phosphorylate $p47^{phox}$ [Benna et al., 1996; Kim and Dinauer, 2001]. On the other hand, we have reported previously that PMA-stimulated PMNs release a much

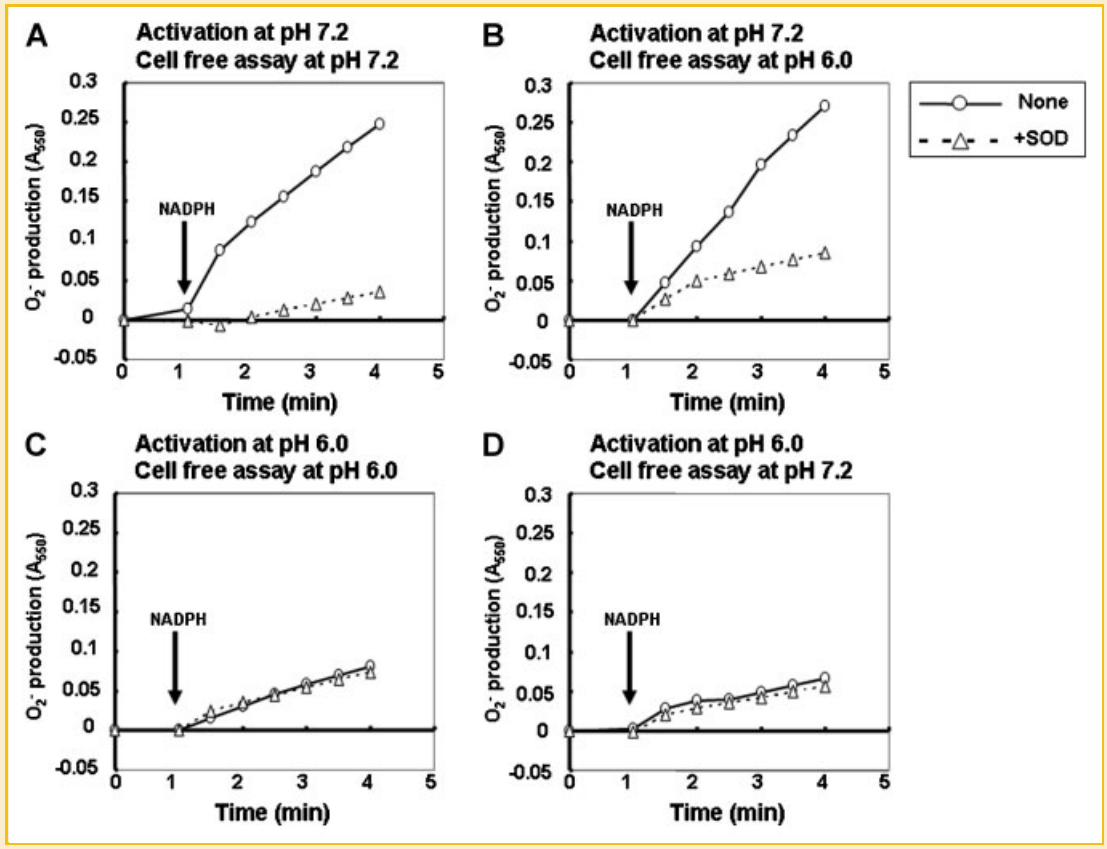

Fig. 6. Time courses of O_2^- production by suspended plasma membrane fractions under cell-free conditions. PMNs in suspension were exposed to PMA for 10 min at pH 7.2 (A and B) or pH 6.0 (C and D), then the plasma membrane fraction was prepared and re-suspended in disruption buffer at pH 7.2 (A and D) or pH 6.0 (B and C), as described in the Materials and Methods section. The cell-free assay mixture consisted of 4.5×10^7 cell equivalents/ml plasma membrane suspension, 50 μM cytochrome *c*, 100 μg/ml catalase and 2 mM NaN_3 in disruption buffer (pH 7.2 or 6.0). One minute after the start of monitoring of the absorbance at 550 nm (A_{550}), 40 μM β-NADPH was added to initiate the enzyme reaction, and monitoring of A_{550} was continued for up to 4 min. The reaction was performed in the absence (clear circles/solid lines) or presence (clear triangles/dashed lines) of 500 U/ml SOD. Data shown are representative of two independent experiments.

lower amount of H_2O_2 at pH 6.0 than at pH 7.2 [Suzuki et al., 2008]. As shown in Figure 5A, although PMNs stimulated with PMA in suspension produced O_2^- at both pH 7.2 and 6.0, the activity at pH 6.0 was much lower than at pH 7.2. Neither SB203580 nor PD98059 inhibited PMA-induced O_2^- production, irrespective of pH (Fig. 5B). To examine whether pre-activated NADPH oxidase complex produced O_2^- at pH 6.0, a cell-free assay of O_2^- production using plasma membrane fractions of PMNs stimulated with PMA at pH 7.2 or 6.0 was performed at each pH. A plasma membrane solution (pH 7.2 or 6.0) was prepared as described in Materials and Methods section, and O_2^- production was initiated by addition of NADPH and monitored as SOD-inhibitable reduction of cytochrome *c*. The results are shown in Figure 6. When the plasma membranes of PMNs stimulated with PMA at pH 7.2 had been dissolved at the same pH, large amounts of O_2^- were produced (Fig. 6A). Importantly, marked O_2^- production was observed when membrane samples had been prepared from PMNs stimulated with PMA at pH 7.2 and the assay was performed at pH 6.0 (Fig. 6B). On the other hand, membrane samples from PMNs stimulated with PMA at pH 6.0 showed no O_2^- production activity when the assay was performed at either pH 6.0 or 7.2 (Fig. 6C,D, respectively).

DISCUSSION

In the present study, we have assessed the effect of acidic pH on O_2^- production resulting from activation of NADPH oxidase after induction of spreading in PMNs.

Figure 1C–F shows that PMNs were able to spread over type I collagen and plastic surfaces, not only at pH 7.2 but also at pH 6.0. In agreement with other reports [Wright et al., 1989; Garnotel et al., 1995; Sud'ina et al., 2001; Kori et al., 2009], spreading over type I collagen surfaces, but not over plastic surfaces, was integrin-dependent because the former, but not the latter, required divalent cations. However, PMNs were shown to be capable of producing O_2^- only at pH 7.2 and in a divalent cation-dependent manner, suggesting that spreading-stimulated activation of NADPH oxidase requires both integrin-mediated signaling and a non-acidic pH environment. As expected from our previous study [Suzuki and Namiki, 2009], PMNs underwent spreading and subsequent O_2^- production only at pH 7.2, but not at pH 6.0, when they had been plated onto fibrinogen surfaces (Fig. 1A,B). Because PMN spreading over fibrinogen surfaces has been reported to be strictly dependent on β_2 integrins [Ugarova et al., 1998; Hu et al., 2001; Mosesson,

2005], and PMNs could not undergo spreading over such surfaces at pH 6.0, it seems that β_2 integrins cannot be activated at acidic pH. However, it is too early to draw a definite conclusion, since integrin $\alpha_M\beta_2$ is capable of recognizing multiple ligands including type I collagen and different denatured proteins with different residues within the α_MI-domain [Sud'ina et al., 2001; Ustinov and Plow, 2002]. The results obtained in our experiments using type I collagen and plastic surfaces (Fig. 1D,F, respectively) suggested that signaling pathways for activation of NADPH oxidase, rather than integrin signaling per se, may be impaired at acidic pH. Our observation that the spreading-inducing ability of fibrinogen surfaces was substantially weak, and that cellular stimulation with H_2O_2, a known activator of β_2 integrins [Bouaouina et al., 2004], was required for induction of spreading that was comparable to that on the other two surfaces we tested, may indicate the presence of a relatively small number of epitopes, or their low affinity, for target integrin molecules on fibrinogen surfaces. The roles of each integrin family in the induction of spreading and O_2^- production remain to be investigated.

The involvement of the p38 MAPK pathway in spreading and subsequent activation of NADPH oxidase in PMNs has been a subject of considerable controversy and confusion in the literature [Detmers et al., 1998; Lynch et al., 1999; Tandon et al., 2000; Bouaouina et al., 2004; Lavigne et al., 2006; Kilpatrick et al., 2010]; although all research groups so far have concluded that p38 MAPK has an indispensable role in adhesion-dependent O_2^- production by PMNs, the mechanisms of p38 MAPK-mediated regulation they have proposed have differed. Several groups have demonstrated that SB203580 inhibits the adhesion of PMNs, resulting in suppression of O_2^- production [Detmers et al., 1998; Bouaouina et al., 2004; Lavigne et al., 2006]. Tandon et al. [2000] have reported that p38 MAPK mediates β_2 integrin up-regulation, contributing to promotion of adhesion in TNF-α-stimulated PMN. In contrast, Lynch et al. [1999] have reported that SB203580 suppresses the activation of NADPH oxidase in eosinophils without any inhibition of cell adhesion. Recently, Kilpatrick et al. [2010] have reported that p38 MAPK becomes associated with $p47^{phox}$ following spontaneous adhesion of PMNs to fibronectin-coated surfaces, and is required for O_2^- production by the adherent PMNs upon stimulation with TNF. The results of our experiments using SB203580 clearly show that spreading-dependent O_2^- production requires the activity of p38 MAPK (Fig. 3). However, the effects of the inhibitor on spreading per se differed among the surfaces onto which PMNs had been plated: Strong inhibition of H_2O_2-stimulated PMN spreading was observed over fibrinogen surfaces, slight attenuation of spontaneous spreading was observed on type I collagen surfaces, and no effect on spreading was evident on plastic surfaces (Fig. 3A, B,C, respectively). The effect of SB203580 we observed in experiments in which H_2O_2-stimulated PMNs had been plated onto fibrinogen surfaces (Fig. 3A) was consistent with some reports [Detmers et al., 1998; Tandon et al., 2000; Bouaouina et al., 2004; Lavigne et al., 2006] but not with others [Lynch et al., 1999; Kilpatrick et al., 2010], while the effects in the other situations were consistent with the latter [Lynch et al., 1999; Kilpatrick et al., 2010] but not with the former [Detmers et al., 1998; Tandon et al., 2000; Bouaouina et al., 2004; Lavigne et al., 2006] (Fig. 3B,C). One possible explanation for our observations is that p38 MAPK may have a dual role of mediating both H_2O_2-induced spreading and activation of NADPH oxidase. This proposal is based on an important observation by Bouaouina et al. [2004] that β_2 integirn activation in PMNs stimulated with TNF-α or H_2O_2 is mediated by redox-regulated activation of p38 MAPK. Three other groups have also demonstrated the involvement of p38 MAPK in the process of adhesion by using PMNs stimulated with TNF-α [Detmers et al., 1998; Tandon et al., 2000; Lavigne et al., 2006]. In our experiments, SB203580 may have possibly inhibited the spreading-promoting effect of H_2O_2 on PMNs plated onto fibrinogen surfaces. Bouaouina et al. [2004] further examined the effect of SB203580 on PMN adhesion directly promoted by the anti-CD18-activating mAb KIM185, requiring no inside-out signaling, and found that p38 MAPK was not involved in the post-binding events necessary for strong adhesion. Their finding is also consistent with our present results showing that spontaneous spreading of PMNs over type I collagen or plastic surfaces was largely resistant to treatment with SB203580. Studies of PMNs in suspension stimulated with TNF-α, LPS, and bacteria have shown that NADPH oxidase activation is primarily mediated by activation of p38 MAPK, but not ERK [McLeish et al., 1998; Ward et al., 2000; Forsberg et al., 2001]. Our results suggest that spreading-dependent activation of NADPH oxidase in PMNs may also substantially require activation of p38 MAPK rather than ERK. Although the molecules that are targeted by the activated p38 MAPK during NADPH oxidase activation signaling in spread PMNs still remain to be identified, p38 MAPK may be involved in the priming of NADPH oxidase by phosphorylating $p47^{phox}$, as reported by Dang et al. [2006].

One of the most important and novel findings of the present study was that integrin-mediated spreading-dependent phosphorylation of p38 MAPK at Thr-180 and Tyr-182, a hallmark of activation [Raingeaud et al., 1995], as well as subsequent O_2^- production, was reversibly down-regulated by environmental acidification (Fig. 4). We demonstrated for the first time that divalent cation-dependent phosphorylation of p38 MAPK in PMNs was markedly promoted when the cells had been incubated for 30 min at pH 7.2, but not at pH 6.0 (Fig. 3D). The data shown in Figure 4B, however, suggest that phosphorylation of p38 MAPK in PMNs was regulated by changes in environmental pH even after induction of spreading, and would have been independent of the pH at which the cells underwent spreading. The reversible regulation of p38 MAPK phosphorylation in PMNs in response to changes in environmental pH after induction of spreading would lead to reversible regulation of NADPH oxidase activation, as shown in Figure 4A.

Although the kinetics of p38 MAPK phosphorylation were not investigated in the present study, phosphorylated p38 MAPK appeared to be stable for more than 30 min, because p38 MAPK was found to have been already phosphorylated in PMNs at 30 min after plating at pH 7.2 (Fig. 3D); also, in separate experiments, such phosphorylation was also detected when PMNs had been incubated for a total of 60 min at pH 7.2 with one refreshment of the culture medium (Fig. 4B). As shown in Figure 2, we found that spreading-dependent O_2^- production by PMNs at pH 7.2 was time-dependent and continued for up to 120 min after induction of spreading when they were plated onto each of the surfaces, followed by incubation

for 30 min, being consistent with previous reports [Berton et al., 1992; Williams and Solomkin, 1999; Kilpatrick et al., 2010] that NADPH oxidase remains in an activated state for a long time. The causal relationship between the stability of phosphorylated p38 MAPK and prolonged activation of NADPH oxidase in spread PMNs would be a potentially faithful area of future study.

In the present (Fig. 5A) and previous [Suzuki et al., 2008] studies, we showed that the PKC-dependent production of O_2^- or H_2O_2 by PMNs stimulated with PMA was largely, but not completely, inhibited at pH 6.0. We found that PMA-induced activation of NADPH oxidase was independent of p38 MAPK or Erk at both pH 7.2 and 6.0 (Fig. 5B), although it has been reported that p38 MAPK is activated by stimulation of PMNs with PMA [Benna et al., 1996]. These results suggest that, at least in the spreading-independent activation of NADPH oxidase induced by PMA, a pH-sensing regulator of NADPH oxidase other than the pathway leading to activation of p38 MAPK also exists. In this connection, using subcellular fractionation and immunoblot analyses, we have observed that the subcellular distribution of PKCβII and δ to the plasma membranes in unstimulated PMNs was promoted by environmental alkalization, but suppressed by its acidification, despite the fact that PMA stimulation resulted in pH-independent complete translocation of PKCs to the membranes [Suzuki and Namiki, 2007]. We also found that PMA-induced membrane translocation of $p47^{phox}$ was barely detectable at acidic pH, but that PMA stimulation induced a PKC-dependent redistribution of the molecule to the non-plasma-membrane "particle" fraction containing nuclei and associated large structures, which was precipitated by low-speed centrifugation of the PMN homogenates [Suzuki et al., 2008]. Therefore, the process of PKC-mediated $p47^{phox}$ translocation may also represent a point at which the NADPH oxidase system is regulated by pH-sensing. Qian et al. [2009] have recently reported that MAPK phosphatase 5 (MKP5), which negatively regulates p38 MAPK, has a non-redundant function in restraining p38 MAPK-mediated activation of NADPH oxidase in PMNs, and that MKP5-deficient PMNs exhibit enhanced O_2^- production in response to different stimuli, including PMA, suggesting that p38 MAPK may function as a positive regulator of NADPH oxidase in PMNs stimulated with PMA. At present, these issues remain to be explored.

Our present findings raise the question of whether the O_2^--producing activity of the activated NADPH oxidase complex is substantially low at acidic pH, and whether instability of O_2^- at acidic pH would affect the reduction of cytochrome *c* by O_2^-. However, these possibilities were ruled out by the cell-free assay of NADPH oxidase using plasma membrane fractions from PMNs stimulated with PMA (Fig. 6). Our results thus clearly indicate that an acidic environment inhibits the process of NADPH oxidase activation, but not the O_2^- production activity of the enzyme.

A pH-sensing regulation system for spreading-dependent activation of NADPH oxidase in PMNs may operate when activated PMNs generate large amounts of intracellular acid equivalents by NADPH oxidase-mediated oxidation of NADPH, contributing to PMN cytoplasmic acidification. Moreover, it has recently been reported that diffusion of HOCl, produced by myeloperoxidase, into the cytosol leads to cytoplasmic acidification in PMNs [Morgan et al., 2009]. Although PMNs possess a number of H^+ extrusion mechanisms to avoid cytoplasmic acidification, the acidified environment in inflammatory tissues tends to impede these systems, resulting in severe cytoplasmic acidification [Grinstein et al., 1991; Suzuki et al., 2008]. On the basis of our present results, we propose that the p38 MAPK-mediated signaling pathway should function as a pH-sensing regulator of NADPH oxidase in PMNs induced to undergo integrin-mediated spreading, in order to avoid tissue-damaging hyperactivation of PMNs. The regulation system may also be important in living animals, since in vivo studies have also shown that p38 MAPK is implicated in PMN NADPH oxidase-mediated tissue injury [Tojo et al., 2005; Dang et al., 2006; Qian et al., 2009].

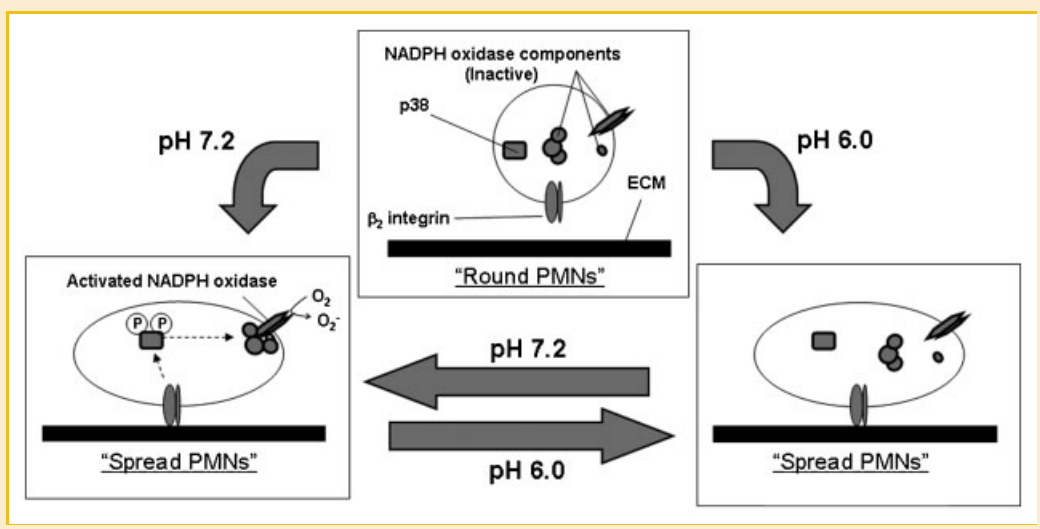

Fig. 7. Simplified schematic representation of our findings. The p38 MAPK-mediated signaling pathway functions as a pH-sensing reversible regulator of NADPH oxidase in PMNs induced to undergo integrin-mediated spreading. O_2^- production is reversibly suppressed by environmental acidification, as a consequence of reversible inhibition of the signaling pathway leading to phosphorylation/activation of p38 MAPK. On immobilized fibrinogen surfaces, however, PMNs are even unable to undergo spreading in an acidified environment, because "pH-sensitive p38 MAPK" is required for induction of spreading (not shown in this diagram).

In conclusion, in PMNs undergoing integrin-mediated spreading, O_2^- production is reversibly suppressed by environmental acidification, as a consequence of reversible inhibition of the signaling pathway leading to activation of p38 MAPK, which is required for spreading-dependent activation of NADPH oxidase (Fig. 7). Environmental neutralization leads to recovery of p38 MAPK-mediated signaling and reactivates the NADPH oxidase. Acidic pH does not affect the O_2^- production activity of pre-activated NADPH oxidase. The p38 MAPK-mediated signaling pathway is thought to function as a pH-sensing regulation system for spreading-dependent activation of NADPH oxidase in PMNs.

REFERENCES

Ago T, Nunoi H, Ito T, Sumimoto H. 1999. Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein $p47^{phox}$. Triple replacement of serines 303, 304 and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in $p47^{phox}$, thereby activating the oxidase. J Biol Chem 274:33644–33653.

Araki A, Inoue T, Cragoe EJ, Jr, Sendo F. 1991. Na^+/H^+ exchange modulates rat neutrophil mediated tumor cytotoxicity. Cancer Res 51:3212–3216.

Benna JE, Han J, Park JW, Schmid E, Ulevitch RJ, Babior BM. 1996. Activation of p38 in stimulated human neutrophils: Phosphorylation of the oxidase component $p47^{phox}$ by p38 and ERK but not by JNK. Arch Biochem Biophys 334:395–400.

Berton G, Laudanna C, Sorio C, Rossi F. 1992. Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. J Cell Biol 116:1007–1017.

Bokoch GM, Knaus UG. 2003. NADPH oxidases: Not just for leukocytes anymore! Trends Biochem Sci 28:502–508.

Bouaouina M, Blouin E, Halbwachs-Mecarelli L, Lesavre P, Rieu P. 2004. TNF-induced β_2 integrin activation involves Src kinases and a redox-regulated activation of p38 MAPK. J Immunol 173:1313–1320.

Chen H, Mocsai A, Zhang H, Ding RX, Morisaki JH, White M, Rothfork JM, Heiser P, Colucci-Guyon E, Lowell CA, Gresham HD, Allen PM, Brown EJ. 2003. Role of plastin in host defense distinguishes integrin signaling from cell adhesion and spreading. Immunity 19:95–104.

Clemens RA, Newbrough SA, Chung EY, Gheith S, Singer AL, Koretzky GA, Peterson EJ. 2004. PRAM-1 is required for optimal integrin-dependent neutrophil function. Mol Cell Biol 24:10923–10932.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which id stimulated by cellular stresses and interleukin-1. FEBS Lett 364:229–233.

Dang PMC, Stensballe A, Boussetta T, Raad H, Dewas C, Kroviarski Y, Hayem G, Jensen ON, Gougerot-Pocidalo MA, El-Benna J. 2006. A specific $p47^{phox}$-serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. J Clin Invest 116:2033–2043.

DeMali KA, Wennerberg K, Burridge K. 2003. Integrin signaling to the actin cytoskeleton. Curr Opin Cell Biol 15:572–582.

Demaurex N, Downey GP, Waddell TK, Grinstein S. 1996. Intracellular pH regulation during spreading of human neutrophils. J Cell Biol 133:1391–1402.

Detmers PA, Zhou D, Polizzi E, Thieringer R, Hanlon WA, Vaidya S, Bansal V. 1998. Role of stress-activated mitogen-activated protein kinase (p38) in β_2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. J Immunol 161:1921–1929.

Forsberg M, Löfgren R, Zheng L, Stendahl O. 2001. Tumor necrosis factor-α potentiates CR3-induced respiratory burst by activating p38 MAP kinase in human neutrophils. Immunology 103:465–472.

Garnotel R, Monboisse JC, Randoux A, Haye B, Borel JP. 1995. The binding of type I collagen to lymphocyte function-associated antigen (LFA) I integrin triggers the respiratory burst of human polymorphonuclear neutrophils. Role of calcium signaling and tyrosine phosphorylation of LFA 1. J Biol Chem 270:27495–27503.

Graham DB, Robertson CM, Bautista J, Mascarenhas F, Diacovo MJ, Montgrain V, Lam SK, Cremasco V, Dunne WM, Faccio R, Coopersmith CM, Swat W. 2007. Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice. J Clin Invest 117:3445–3452.

Grinstein S, Swallow CJ, Rotstein OD. 1991. Regulation of cytoplasmic pH in phagocytic cell function and dysfunction. Clin Biochem 24:241–247.

Hu WJ, Eaton JW, Tang L. 2001. Molecular basis of biomaterial-mediated foreign body reactions. Blood 98:1231–1238.

Kilpatrick LE, Sun S, Li H, Vary TC, Korchak HM. 2010. Regulation of TNF-induced oxygen radical production in human neutrophils: Role of δ-PKC. J Leukoc Biol 87:153–164.

Kim C, Dinauer MC. 2001. Rac 2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. J Immunol 166:1223–1232.

Kori S, Namiki H, Suzuki K. 2009. Biphasic regulation of polymorphonuclear leukocyte spreading by polyphenolic compounds with pyrogallol moieties. Int Immunopharmacol 9:1159–1167.

Lavigne LM, Albina JE, Reichner JS. 2006. β-Glucan is a fungal determinant for adhesion-dependent human neutrophil functions. J Immunol 177:8667–8675.

Lowell CA, Berton G. 1999. Integrin signal transduction in myeloid leukocytes. J Leukoc Biol 65:313–320.

Lynch OT, Giembycz MA, Barnes PJ, Hellewell PG, Lindsay MA. 1999. 'Outside-in' signaling mechanisms underlying CD11b/CD18-mediated NADPH oxidase activation in human adherent blood eosinophils. Br J Pharmacol 128:1149–1158.

McLeish KR, Klein JB, Coxon PY, Head KZ, Ward RA. 1998. Bacterial phagocytosis activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in human neutrophils. J Leukoc Biol 64:835–844.

Morgan D, Capasso M, Musset B, Cherny VV, Ríos E, Dyer MJ, DeCoursey TE. 2009. Voltage-gated proton channels maintain pH in human neutrophils during phagocytosis. Proc Natl Acad Sci USA 106:18022–18027.

Mosesson MW. 2005. Fibrinogen and fibrin structure and functions. J Thromb Haemost 3:1894–1904.

Nasmith PE, Grinstein S. 1986. Impairment of Na^+/H^+ exchange underlies inhibitory effects of Na^+-free media on leukocyte function. FEBS Lett 202:79–85.

Nathan C, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright SD. 1989. Cytokine-induced respiratory burst of human neutrophils: Dependence on extracellular matrix proteins and CD11/CD18 integrins. J Cell Biol 109:1341–1349.

Nick JA, Avdi NJ, Young SK, Knall C, Gerwins P, Johnson GL, Worthen GS. 1997. Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. J Clin Invest 99:975–986.

Pluskota E, Soloviev DA, Szpak D, Weber C, Plow EF. 2008. Neutrophil apoptosis: Selective regulation by different ligands of integrin $\alpha_M\beta_2$. J Immunol 181:3609–3619.

Qian F, Deng J, Cheng N, Welch EJ, Zhang Y, Malik AB, Flavell RA, Dong C, Ye RD. 2009. A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury. EMBO J 28:2896–2907.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270:7420–7426.

Roos D, de Boer M, Kuribayashi F, Meichl C, Weening RS, Segal AW, Ahlin A, Nemet K, Hossle JP, Bernatowska-Matuszkiewicz E, Middleton-Price H. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granuromatous disease. Blood 87:1663–1681.

Sawyer RG, Spengler MD, Adams RB, Pruett TL. 1991. The peritoneal environment during infection. The effect of monomicrobial and polymicrobial bacteria on pO_2 and pH. Ann Surg 213:253–260.

Sud'ina GF, Brock TG, Pushkareva MA, Galkina SI, Turutin DV, Peters-Golden M, Ullrich V. 2001. Sulphatides trigger polymorphonuclear granulocyte spreading on collagen-coated surfaces and inhibit subsequent activation of 5-lipoxygenase. Biochem J 359:621–629.

Suzuki K, Kori S, Morikawa M, Takagi A, Namiki H. 2010. Oxidative stress-mediated bimodal regulation of polymorphonuclear leukocyte spreading by polyphenolic compounds. Int Immunopharmacol 10:1448–1455.

Suzuki K, Kosho I, Namiki H. 2008. Characterization of the unique regulatory mechanisms of phorbol ester-induced polymorphonuclear leukocyte spreading in an acidified environment. Eur J Pharmacol 588:301–308.

Suzuki K, Namiki H. 2007. Cytoplasmic pH-dependent spreading of polymorphonuclear leukocytes: Regulation by pH of PKC subcellular distribution and F-actin assembly. Cell Biol Int 31:279–288.

Suzuki K, Namiki H. 2009. Proteolysis of fibrinogen deposits enables hydrogen peroxide-stimulated polymorphonuclear leukocytes to spread in an acidified environment. Eur J Pharmacol 609:140–147.

Tandon R, Sha'afi RI, Thrall RS. 2000. Neutrophil β2-integrin upregulation is blocked by a p38 MAP kinase inhibitor. Biochem Biophys Res Commun 270:858–862.

Tojo A, Onozato ML, Kobayashi N, Goto A, Matsuoka H, Fujita T. 2005. Antioxidative effect of p38 mitogen-activated protein kinase inhibitor in the kidney of hypertensive rat. J Hypertens 23:165–174.

Ugarova TP, Solovjov DA, Zhang L, Loukinov DI, Yee VC, Medved LV, Plow EF. 1998. Identification of a novel recognition sequence for integrin $\alpha_M\beta_2$ within the γ-chain of fibrinogen. J Biol Chem 273:22519–22527.

Ustinov VA, Plow EF. 2002. Delineation of the key amino acids involved in neutrophil inhibitory factor binding to the I-domain supports a mosaic model for the capacity of integrin $\alpha_M\beta_2$ to recognize multiple ligands. J Biol Chem 277:18769–18776.

Ward RA, Nakamura M, McLeish K. 2000. Priming of the neutrophil respiratory burst involves p38 mitogen-activated protein kinase-dependent exocytosis of flavocytochrome b_{558}-containing granules. J Biol Chem 275:36713–36719.

Williams MA, Solomkin JS. 1999. Integrin-mediated signaling in human neutrophil functioning. J Leukoc Biol 65:725–736.

Wright SD, Levin SM, Jong MTC, Chad Z, Kabbash LG. 1989. CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. J Exp Med 169:175–183.

Yuo A, Kitagawa S, Azuma E, Natori Y, Togawa A, Saito M, Takaku F. 1993. Tyrosine phosphorylation and intracellular alkalinization are early events in human neutrophils stimulated by tumor necrosis factor, granulocyte-macrophage colony-stimulating factor and granurocyte colony-stimulating factor. Biochim Biophys Acta 1156:197–203.

Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, Huang CK, Sha'afi RI. 1998. p38 Mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-α or FMLP stimulation. J Immunol 160:1982–1989.