Nitric Oxide Produced in Response to Engagement of β2 Integrins on Human Neutrophils Activates the Monomeric GTPases Rap1 and Rap2 and Promotes Adhesion*

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We found that engagement of β2 integrins on human neutrophils increased the levels of GTP-bound Rap1 and Rap2. Also, the activation of Rap1 was blocked by PP1, SU6656, LY294002, GF109203X, or BAPTA-AM, which indicates that the downstream signaling events in Rap1 activation involve Src tyrosine kinases, phosphoinositide 3-kinase, protein kinase C, and release of calcium. Surprisingly, the β2 integrin-induced activation of Rap2 was not regulated by any of the signaling pathways mentioned above. However, we identified nitric oxide as the signaling molecule involved in β2 integrin-induced activation of Rap1 and Rap2. This was illustrated by the fact that engagement of β2 integrins increased the production of nitrite, a stable end-product of nitric oxide. Furthermore, pretreatment of neutrophils with Nω-monomethyl-L-arginine, or 1400W, which are inhibitors of inducible nitric-oxide synthase, blocked β2 integrin-induced activation of Rap1 and Rap2. Similarly, Rp-8pCPT-cGMPS, an inhibitor of cGMP-dependent serine/threonine kinases, also blunted the β2 integrin-induced activation of Rap GTPases. Also nitric oxide production and its downstream activation of cGMP-dependent serine/threonine kinases were essential for proper neutrophil adhesion by β2 integrins. Thus, we made the novel findings that β2 integrin engagement on human neutrophils triggers production of nitric oxide and its downstream signaling is essential for activation of Rap GTPases and neutrophil adhesion.

The β2 integrins constitute the main group of adhesion molecules expressed on polymorphonuclear neutrophils (PMNs), and they play an essential role in adhesion of these cells to the vascular endothelium during the inflammatory response and subsequent diapedesis from post-capillary venules into tissues. These transmembrane receptors are heterodimeric proteins composed of a common β chain (CD18) and one of four unique α chains (designated CD11 a–d), with CD11b/CD18 being the most prominent β2 integrin on PMNs (1). The signal transduction properties of these integrins are crucial for regulation of several PMN functions. In human PMNs, adhesion-induced ligation of the β2 integrins activates various non-receptor tyrosine kinases, such as Src family members (p58c-src (Fgr), p59/61hck (Hck)), and p53/56lyn (Lyn)), the non-Src tyrosine kinases p72Syk (Syk), and the proline-rich tyrosine kinase Pyk2 (2). Genetic studies of knock-out mice have shown that the Src tyrosine kinases Fgr, Hck, and Lyn (3), as well as Syk (4), are necessary for β2 integrin-dependent modulation of a number of functions in PMNs. In the cited investigations, adhesion-induced activation of the respiratory burst and degranulation were impaired in PMNs from Fgr−/− Hck−/− (3) or Syk−/− (4) mice, as revealed by the finding that those cells displayed defective adhesion and spreading on various types of biological surfaces. Using another strategy, Fuortes and coworkers (5) have elegantly demonstrated that Pyk2 is involved in tumor necrosis factor-α (TNF-α)-induced PMNs spreading and activation of the respiratory burst but not in degranulation. Other key signaling enzymes that are also activated upon engagement of the PMN β2 integrins include phosphoinositide (PI) 3-kinase (6) and phospholipase C (PLC) (7). PLC acts via production of diacylglycerol and inositol triphosphate to stimulate membrane fusion and the respiratory burst in neutrophils.

The abbreviations used are: PMNs, polymorphonuclear neutrophils; cGKI, cGMP-dependent protein kinase; cGKI I, cGKI inhibitor; MPO, myeloperoxidase; L-NMMA, Nω-nitro-L-arginine; NO, nitric oxide; NOS, nitric-oxide synthase; PI, phosphoinositide; PKC, protein kinase C; PP1, 4-amino-5-(4-methylphenoxy)-7-(1-butylpiperazino)-3,4-dipyrimidine; Rp-8pCPT-cGMPS, 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphothioate; R0, isomer tetraethylammonium salt; TFN, tumor necrosis factor; GST, glutathione S-transferase; PLC, phospholipase C; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N′,N′-tetraacetic; PBS, phosphate-buffered saline; RBD, Rap1-binding domain; fMLP, formylmethionylleucylphenylalanine; NS, not significant.

* This work was supported in part by grants from the Swedish Cancer Foundation and the Swedish Medical Research Council (to T. A.); by the Crafoord Foundation, the Johan and Greta Kocks Foundation, and the Royal Physiographical Society in Lund (to K. D.); and by the Osterlund Foundation, the Universitetsjukhus-Malmö Ålmanna Sjukhus Research Foundations, and the Blood and Defense Network of Lund University (to T. A. and K. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Involved in a cost-sharing program between the Swedish Institute, and the Hungarian Academy of Sciences, and partly supported by the Swedish Institute; the European Union (project of the European Union for Centre of Excellence in Biomolecular Chemistry, QLK2-CT-2002-90436); and the Faculty of Medicine and Health Sciences, Queen’s University Belfast (Friel funds).

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cytosolic free Ca\(^{2+}\) (9), respectively, which is essential for activation of several PMN functions.

Nitric oxide (NO) is a gaseous signaling molecule involved in host defense and immune responses (10). NO is a short-lived free radical that is produced by nitric-oxide synthases (NOSs) from L-arginine, O\(_2\), and NADPH-derived electrons (11). NO is capable of diffusing across membranes and reacting with transition metals (iron, zinc, and copper), which are part of the prosthetic groups of enzymes. Thereby, NO regulates the activity of various enzymes, including soluble guanylate cyclases. Soluble guanylate cyclases are rapidly activated by low concentrations of NO (10–100 nM) and produce cGMP from GTP, which in turn binds to and activates cGMP-dependent serine/threonine protein kinases (cGKI) (11).

It is still controversial as to whether there is an enzymatic production of NO in human PMNs (11). Nonetheless, several lines of evidence suggest that NO plays an important role in regulating PMN functions. For instance, human peripheral PMNs express inducible NO synthase (iNOS) (12) and neuronal NO synthase (13), and levels of iNOS are augmented during bacterial infection (14) or TNF-induced PMN adhesion (15). Furthermore, PMNs produce NO in response to extracellular stimuli (16–18) and express type I cGKI (19). The regulatory role of NO in PMNs seems to be complex and dependent on its concentration. Thus, high doses of NO or cGMP analogues inhibit chemotaxis, whereas lower concentrations facilitate this response (20).

Activation of PMN functions as well as leukocyte recruitment in tissues, induced by pro-inflammatory cytokines, is tightly dependent on PMN adhesion (21). The fact that NO regulates leukocyte accumulation in pathological states (11) indicates that this gaseous signaling molecule might control PMN adhesion.

In leukocytes, the monomeric GTPase Rap1 has emerged as an important regulator of cell adhesion through its ability to cause functional activation of \(\beta2\) integrins. For example, it has been shown that expression of a dominant-negative Rap1 in macrophages prevents activation of the \(\beta2\) integrin CD11b/CD18 by TNF, and, as a consequence, cell adhesion is prevented (22).

Rap GTPases belong to the Ras superfamily of small GTP-binding proteins. The Rap subfamily has four members designated Rap1A, Rap1B, Rap2A, and Rap2B (23), of which Rap1A and Rap2B are the major Rap GTPases expressed in PMNs (24, 25). A hallmark of small GTP-binding proteins is their ability to dynamically cycle between an inactive GDP-bound and an active GTP-bound state (26). The GDP-GTP switch is brought about by guanine nucleotide exchange factors, which are activated by extracellular stimuli. Several guanine nucleotide exchange factors for Rap have been identified that can be stimulated by different signaling pathways (27), but the Rap guanine nucleotide exchange factors that are expressed in PMNs have not yet been characterized. It is in the GTP-bound state that these proteins interact with specific effectors to initiate downstream signals and functions. Subsequently, a GTPase-activating protein is responsible for the inactivation of these GTP-binding proteins by catalyzing the hydrolysis of bound GTP to GDP.

In the present work, we aimed to determine whether \(\beta2\) integrins can activate Rap GTPases, similar to their previously demonstrated ability to regulate other members of the Ras GTPase superfamily (28–30), and, if that proved to be true, to identify which signaling pathways are implicated in the regulation of these GTPases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-Rap1 (Ref.: 610195) and anti-Rap2 (Ref.: 610215) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences Pharmingen, respectively. IB4 mAb (mouse anti-human CD18, IgG\(_2\)a isotype) was originated from Dr. S. Wright (Rockefeller University, New York, NY) (31); the negative control mAb IgG\(_2\)a Ab (Ref.: X0943) (directed against Aspergillus niger glucose oxidase) was from DakoCytomation (Glostrup, Denmark); and the fluorescein isothiocyanate (FITC) anti-human CD18 Ab (clone CLB-0943) (directed against Aspergillus niger glucose oxidase) was from eBioscience (Wembley, UK). The PI 3-kinase inhibitor LY294002, the nitric-oxide synthase inhibitor \(\text{N}^\text{ω}\)-monomethyl-L-arginine (\(\text{L}-\text{NMMA}\)) (32), the Src family tyrosine kinase inhibitor SU6656 (33), and benzamidine were obtained from Sigma-Aldrich; the Src family inhibitor PP1 (34) and \(R_\text{g}^{-8}\text{pCPT-cGMPS}, the inhibitor of cGMP-dependent protein kinases (cGKI I) (Ref.: ALX-480-032) (35), were from Alexis/Axxora (Nottingham, UK); BAPTA-AM and the PKC inhibitor GF-109203X (36) were from Calbiochem; the inducible nitric-oxide synthase inhibitor 1400W (37) was from Cayman Chemicals (Stockholm, Sweden); dextran and Ficoll-Hypaque were from Amersham Biosciences. We purchased the protease inhibitors Pefabloc, pepstatin, leupeptin, aprotinin, and antipain from Roche Applied Science, and all electrophoresis reagents from Bio-Rad. The FITC isotype control mouse IgG\(_1\) (Ref.: F-6397) as well as all other chemicals (of analytical grade) came from Sigma-Aldrich.

**Isolation of Human PMNs**—Blood from healthy donors was collected and isolated under endotoxin-free conditions as previously described (38), and the cells were resuspended in a calcium-containing medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 5.0 mM NaHCO\(_3\), 1.1 mM CaCl\(_2\), 0.1 mM EGTA, 5.5 mM glucose, 20 mM Hepes; pH 7.4). The cell suspension consisted of ~97% of PMNs.

**Engagement of \(\beta2\) Integrins**—Easy Grip(TM) Petri dishes were incubated overnight at 4 °C or for 2 h at room temperature with 20 µg/ml fibrinogen (a ligand for \(\beta2\) integrins) (39) in phosphate-buffered saline (PBS) and then washed twice with PBS and once with calcium-containing medium. PMNs (20 × 10\(^6\)) were subsequently added on fibrinogen-coated plates at 37 °C after which TNF (20 ng/ml) was immediately added to the plates for different periods of time to engage the \(\beta2\) integrins (40). It is well established that incubation of PMNs with TNF increases the level of \(\beta2\) integrins on the surface of the cells (41). In addition, inflammatory mediators such as TNF change the integrins structurally, which converts them from an inactive to an active conformation (42). As a control, suspended PMNs (10\(^3\)/ml) were incubated in polypropylene tubes (15 ml) that had been blocked for 2 h with 10% bovine serum albumin and then rinsed extensively with PBS and once with calcium-containing medium. Incubation of suspended cells was carried out...
under gentle rotation at 37°C in the absence or presence of TNF (20 ng/ml) for the indicated periods of time. An alternative approach used to engage β2 integrins on PMNs entailed incubating the cells on a surface coated with anti-β2 integrin mAbs, in the absence of TNF, as previously described (29, 43). With both methods, ligation of β2 integrins was terminated by placing the Petri dishes or the polypropylene tubes on ice.

Adhesion Assays—Calcine AM is not fluorescent but, once loaded into the cells, is cleaved by endogenous esterases to produce the highly fluorescent dye calcine, which has absorption and emission maxima at 495 and 517 nm, respectively. We pre-incubated PMNs (5 × 10⁶) with 1 μM calcine AM for 30 min at 37°C. Thereafter, the cells were washed, resuspended in calcium-containing medium, and incubated in the absence or presence of PP1 (3 μM), LY294002 (20 μM), GF-109203X (10 μM), EGTA (1 mM), and BAPTA-AM (30 μM), 1,10-phenanthroline (1 μM), 1,10-phenanthroline (1 μM), 1,10-phenanthroline (1 μM), 1400W (10 μM), or Rg-8P-CPT-cGMPS (1 μM). Control PMNs were treated with the vehicle (0.1% Me₂SO or water). Next, the cells were allowed to adhere to fibrinogen-coated, 96-well plates in the presence of TNF (20 ng/ml) for 20 min, after which non-adherent cells were removed, and the wells were washed with calcium-containing medium. The fluorescence in each well was measured (using a fluorescence micro-plate reader) both before and after washing, and the percentage of adherent cells was calculated by dividing the latter value by the former.

PMNs Labeling and Flow Cytometry Analysis of CD18 Expression—PMNs (100,000), resuspended in 100 μl of calcium-containing medium, were stimulated for 20 min with TNF (20 ng/ml) or the vehicle after which, cells were fixed with 1% paraformaldehyde. Cells were subsequently washed twice with the calcium-containing medium, and incubated on ice and in the dark with either the FITC-conjugated anti-human CD18 Ab (1/20 dilution) or the FITC-conjugated isotype control mouse IgG1 (1/20 dilution). After 30 min, cells were spun down, washed twice, and resuspended in 600 μl of the calcium-containing medium. Surface expression of CD18 was analyzed by flow cytometry using a FACScan (BD Biosciences). After selecting gating for PMNs on the forward angle and 90° light scatter plot, histograms indicating mean fluorescence intensity were generated. The data are represented as mean fluorescence intensity.

GST Pull down Assay and Western Blot Analysis—The cDNA of the Rap1-binding domain (RBD) from RaLGDS cloned into a bacterial expression vector was expressed in Escherichia coli as a fusion protein with GST (44). PMNs were lysed in a buffer composed of 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 100 mM NaCl, 10 mM MgCl₂, 5% glycerol, 1 mM Na₃VO₄, and protease inhibitors (20 μg/ml aprotinin; 1 mg/ml each of pepstatin, leupeptin, and antipain; 2.5 mM benzamidine; 2 μM Pefabloc). The GST-RaLGDS-RBD fusion protein was coupled to glutathione-Sepharose beads for 1 h, and then the beads were washed and subsequently added to the clarified PMN lysates. After 1 h, the beads were collected by centrifugation and washed three times in 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM dithiothreitol, 100 mM NaCl, and 30 mM MgCl₂. Next, the beads were resuspended in Laemmli sample buffer and boiled under reducing conditions. The precipitated proteins were subjected to 12% SDS-PAGE and transferred to polyvinyldene difluoride membranes. The membranes were blocked in PBS supplemented with 0.2% Tween 20 and 3% bovine serum albumin (for Rap2) or 5% nonfat dried milk (for Rap1), and then incubated for 1 h with an anti-Rap2 mAb (1/20,000 anti-Rap2) or overnight in a cold room with an anti-Rap1 polyclonal antibody (1/2,000 dilution of anti-Rap1). Thereafter, the membranes were washed in PBS supplemented with 0.2% Tween 20, and subsequently, the membranes were incubated for 1 h with peroxidase-conjugated anti-mouse or anti-rabbit IgGs (1:10,000) in blocking buffer. The blots were washed, and antibody binding was visualized by enhanced chemiluminescence (ECL). For control experiments, GST alone was expressed in E. coli and used to perform pull-down assays as described above.

Nitrite Production—Nitrite production is measured using a nitric oxide quantitation kit (Active Motif, Ede, The Netherlands) (45). Nitrite is quantified by the addition of Griess reagent, which converts nitrite into a purple-colored azo compound. Accurate concentration of nitrite can be determined by photometric measurement of the colored azo compound. Nitrite concentration in the medium is determined by reading the absorbance at 540 nm and referring to a standard curve.

Protein Measurement—The protein content was estimated according to Schaffner and Weissmann (46).

RESULTS

Engagement of β2 Integrins on Human PMNs Induces Activation of Rap GTPases—Our first aim was to examine the previously unaddressed issue of whether Rap1 and Rap2 are regulated by β2 integrins in human PMNs. To engage β2 integrins on PMNs, the cells were incubated on a surface coated with fibrinogen (a ligand for β2 integrins) (39) in the presence of TNF (20 ng/ml) for different periods of time. The relative activities of Rap GTPases were measured by using the GST-RalGDS-RBD binding assay, which is based on the knowledge that the GST-RalGDS-RBD fusion protein binds the active GTP-bound forms of Rap but does not bind inactive GDP-bound Rap (44). The results indicate that engagement of β2 integrins on PMNs increased the amounts of GTP-bound Rap1 and Rap2 in a time-dependent manner, and the highest levels (2- to 3-fold increase over controls) were detected after 20 min of adhesion to a fibrinogen-coated surface (Fig. 1A). Rap2 is visualized as a doublet in the immunoblots, which agrees with previously published data on PMNs (25) and platelets (47). In control experiments, suspended PMNs incubated in the presence of TNF (20 ng/ml) (Fig. 1B, lane 3) or PMNs incubated on fibrinogen-coated plates in the absence of TNF (Fig. 1C, lane 2) exhibited no increase in the basal activity of Rap1 or Rap2. These results confirm that: 1) TNF can only indirectly affect the levels of GTP-bound Rap via up-regulation and activation of β2 integrins on PMNs, and 2) engagement of β2 integrins cannot occur in the absence of TNF. These findings concur well with a previous study showing that TNF-induced activation of selective PMN functions depends on adhesion and ligation of β2 integrins (21).

In control experiments, we established that the GST-RalGDS fusion protein (Fig. 1C, lane 4) but not GST (Fig. 1C, lane 3) bound the active GTP-bound forms of Rap1 and Rap2. Thus we...
Grin-mediated activation of Rap1 but not Rap2

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We have found that pretreatment of PMNs with LY294002 (20 μM) or PP1 (3 μM) partly, but significantly, reduced the β2 integrin-induced loading of Rap1 and Rap2. In contrast, activation of Rap2 brought about in the same manner was not inhibited by LY294002 (20 μM) or PP1 (3 μM) (Fig. 2A, left panel). Because PP1 or LY294002 did only partly block the β2 integrin-induced loading of Rap1 with GTP, we tested whether a combination of PP1 and LY294002 would lead to additive inhibitory effects. We found that addition of LY294002 (20 μM) together with PP1 (3 μM) to leukocytes resulted in a similar level of inhibition than in PMNs pretreated with either PP1 (3 μM) or LY294002 (20 μM) (Fig. 2B). Thus, it is likely that Src tyrosine kinases and PI 3-kinase lie in the same signaling pathway to inhibit Rap1 activation.

**FIGURE 1.** β2 integrin-induced activation of Rap1 and Rap2 in PMNs. A, PMNs (20 × 10^6) were incubated on a surface coated with fibrinogen (20 μg/ml) in the presence of TNF (20 ng/ml) for the indicated time periods. Thereafter, the cells were lysed, and GST-RalGDS-RBD pull-down assays were performed as described under "Experimental Procedures." Proteins bound to GST-RalGDS-RBD were separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and then immunoblotted with an anti-Rap1 Ab (left panel) and Rap2 (right panel). The data are expressed as percentage of unstimulated control PMNs and represent means ± S.E. of 3–6 (for Rap1) and 4 (for Rap2) separate experiments. B and C, PMNs (20 × 10^6) in suspension (Susp), or incubated on a surface coated with fibrinogen (Adh), were (+) or were not (−) exposed to TNF (20 ng/ml) for 20 min. Thereafter, pull-down assays were performed using GST-RalGDS-RBD or GST alone as a control. Levels of active Rap1 and Rap2 were measured as described in A. One representative experiment (out of three) is shown. D, PMNs (20 × 10^6) were incubated for 20 min on surfaces coated with either anti-CD18 (IB4, anti-β2 integrin mAbs) or negative control IgG2a mAbs (in the absence of TNF), after which the cells were lysed. The blots show the amounts of active Rap1 and Rap2 determined by the GST-RalDGS pull-down binding assay, as described above. Statistical significance versus suspended control cells (unpaired Student’s t test) was as follows: *p < 0.05; **p < 0.001; NS, not significant.
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![Image](image)

**FIGURE 2.** PI 3-kinase and Src family tyrosine kinases are involved in β2 integrin-induced activation of Rap1 but not Rap2. Suspended PMNs (20 × 10^6) were pretreated for 20 min at 37 °C with vehicle (0.1% Me₂SO for control cells), PP1 (3 μM) or LY294002 (20 μM, LY) (A) or a combination of PP1 (3 μM) and LY294002 (20 μM, LY) (B). Thereafter, the cells were incubated on a surface coated with fibronectin in the presence of TNF (20 ng/ml) for 20 min, then lysed, and the amounts of GTP-bound active Rap1 (A, left panel, and B) and Rap2 (A, right panel) were measured. The insets show representative Western blots of each Rap GTPase. The diagrams illustrate densitometric analysis of the relative activities of Rap1 and Rap2 in PMNs. The data are expressed as percentage of suspended (time zero) cells and represent means ± S.E. of 10 (A, left panel), 7–9 (A, right panel), or 10 (B) separate experiments. C, suspended PMNs (20 × 10^6) were pretreated for 20 min at 37 °C with vehicle (0.1% Me₂SO for control cells), PP1 (3 μM), or SU6656 (3 μM, SU). Thereafter, the cells were incubated on a surface coated with fibronectin in the presence of TNF (20 ng/ml) for 20 min, after which the cells were lysed, and levels of GTP-bound Rap1 were determined. One representative experiment out of five is shown. D, PMNs (5 × 10^6) were preincubated for 30 min at 37 °C with the fluorescent probe calcine AM (1 μM), after which PM1 (3 μM), LY294002 (20 μM), or a combination of PP1 (3 μM) and LY294002 (20 μM) was added. After 20 min, the cells were collected, washed, and then incubated on 96-well microplates coated with fibronectin and stimulated with TNF (20 ng/ml) for 20 min. Non-adherent cells were subsequently removed by aspiration, and the wells were washed with calcium-containing medium, after which fluorescence of each well was measured using a fluorescence microplate reader. The percentage of adherent cells (y-axis) was calculated by dividing the fluorescence of plates after washing by the fluorescence measured before washing and multiplied by 100. Statistical significance versus corresponding controls (unpaired Student’s t test) was as follows: ***, p < 0.001; NS, not significant.

To further confirm that the inhibitory effect of PP1 on β2 integrin-induced activation of Rap1 was due to inhibition of Src tyrosine kinases, we made use of another specific inhibitor of Src tyrosine kinases, SU6656. The rational for using this inhibitor is that SU6656 is an indolone analogue, which is structurally unrelated to the pyrazolo-pyrimidine PP1. Because their nonspecific effects do not overlap, the use of SU6656 would further support the involvement of Src family kinases in the regulation of Rap1 by β2 integrins. We found that similar to PP1, pretreatment of PMNs with SU6656 (30 min, 3 μM) partly but significantly blocked β2 integrin-induced activation of Rap1 (Fig. 2C).

We also observed that the effects of LY294002 (20 μM) and PP1 (3 μM) on β2 integrin-induced activation of Rap1 could not be ascribed to the ability of those inhibitors to impair adhesion of PMNs to a surface coated with fibrinogen (Fig. 2D). In addition, wortmannin, a PI 3-kinase inhibitor, which is structurally unrelated to LY294002 and blocks FMLP-induced stimulation of PMNs (53), also blocked β2 integrin-induced activation of Rap1 (adherent cells, 100; adherent cells treated with wortmannin, 59 ± 12, n = 6, p < 0.005) but not Rap2 (adherent cells, 100; adherent cells treated with wortmannin, 124 ± 32, n = 6, NS). Because tyrosine kinases are involved in the β2 integrin-induced activation of PLCγ2 in human PMNs (2, 7), the above results might indicate that the Src tyrosine kinase inhibitors PP1 and SU6656 impair activation of Rap1 by β2 integrins through their ability to block PLCγ2 and leading to a subsequent downstream reduction in calcium release and PKC activation.

**Calcium Signaling and PKC Are Involved in β2 Integrin-mediated Activation of Rap1 but Not Rap2**—Engagement of β2 integrins on PMNs is accompanied by a transient rise in cytosolic free calcium (54). Furthermore, it has been shown that Rap1 in human PMNs is activated by exposure to the artificial calcium-mobilizing drugs ionomycin and thapsigargin (44). To verify whether calcium signaling is required for β2 integrin-induced activation of Rap GTPases, we pretreated PMNs with both EGTA (1 mM) and the cell-permeant calcium chelator...
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BAPTA-AM (30 μM) to deplete intracellular Ca\(^{2+}\) stores, an approach known to abolish any agonist-induced changes in cytosolic free Ca\(^{2+}\) levels in PMNs (44). We found that EGTA plus BAPTA-AM significantly impaired the β2 integrin-induced activation of Rap1 (Fig. 3A, left panel) but had no effect on the simultaneous activation of Rap2 (Fig. 3A, right panel).

Inasmuch as earlier research had shown that PKC activity is increased after engagement of β2 integrins on PMNs (55), we next performed experiments to determine whether PKC family members are involved in regulation of Rap GTPases by β2 integrins. We found that pretreatment of PMNs with the broad-range PKC inhibitor GF-109203X (5 μM) completely blocked the β2 integrin-dependent activation of Rap1 (Fig. 3B, left panel) but had no significant impact on adhesion-induced activation of Rap2 (Fig. 3B, right panel). We used GF-109203X at a concentration of 5 μM, which is known to potently inhibit classic and novel PKC isoforms but has no influence on the atypical isoforms (56). We have again ruled out the possibility that the observed inhibitory effects of GF-109203X and EGTA plus BAPTA-AM on β2 integrin-induced activation of Rap1 were due to impaired adhesion of PMNs to a fibrinogen-coated surface (Fig. 3C).

Engagement of β2 Integrins on PMNs Induces Accumulation of Nitrites—In the cell, NO undergoes a series of reactions with several molecules present in biological fluids and is eventually metabolized to nitrite (NO\(_2\)) and nitrate (NO\(_3\)). Therefore, these stable end products of NO can be taken as an index for NO production. The measurement of nitrite by the Griess reagent is the most commonly used method to estimate nitrite release from cells. As shown in Fig. 4A, the concentration of nitrite in the incubation medium of resting PMNs was 0.43 μM. This basal level of nitrite increased in a time-dependent manner upon adhesion of PMNs by β2 integrins. The highest concentration of nitrite in the medium was obtained after 10- to 20-min adhesion on a fibrinogen-coated surface. At these time points, the concentration of nitrite reached 1.5 μM (3.5-fold increase over basal). For longer incubation periods (30 min), the level of nitrite produced by adherent PMNs dropped back to nearly basal levels. Thus, production of nitrite, in response to engagement of β2 integrins on PMNs, coincides with activation of Rap1 and Rap2. We confirmed that production of nitrite was entirely dependent on cell adhesion and engagement of β2 integrin, because nitrite concentrations did not augment in suspended PMNs incubated with TNF (20 ng/ml, Fig. 4B). It must be reported that the level of nitrite produced in response to β2 integrin engagement varied greatly between donors. For example, PMNs isolated from the three donors used to generate the results presented in Fig. 4B produced low levels of nitrites in

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**FIGURE 3. Role of the calcium signal and PKC family members in β2 integrin-induced regulation of Rap1 and Rap2.** Suspended PMNs (20 × 10⁶) were pretreated for 20 min at 37 °C with EGTA (1 mM) and the intracellular calcium chelator BAPTA-AM (30 μM) (A), or with GF-109203X (5 μM) (B). Suspended control cells were treated with the vehicle (0.1% Me₂SO). Thereafter, the leukocytes were incubated on a surface coated with fibrinogen in the presence of TNF (20 ng/ml) for 20 min. The cells were then lysed, and the amounts of active GTP-bound Rap1 (A and B, left panels) and Rap2 (A and B, right panels) in the lysates were measured as described in the legend of Fig. 1. The arrows on the right indicate the positions of Rap1 and Rap2. The diagrams illustrate densitometric analysis of the relative activities of Rap1 and Rap2 in PMNs that had been pretreated with the indicated inhibitors. C, PMNs (5 × 10⁶) were preincubated for 30 min with the fluorescent probe calcein AM (1 μM) in the absence or presence of inhibitors: EGTA (1 mM) plus BAPTA-AM (30 μM) (left panel), or only GF-109203X (5 μM) (right panel). Thereafter, the cells were incubated on a surface coated with fibrinogen in the presence of TNF (20 ng/ml) for 20 min. Non-adherent cells were removed by aspiration, and the percentage of adherent cells was calculated as described in the legend to Fig. 2. The data are expressed as percentage of adherent cells and represent means ± S.E. of four separate experiments. Statistical significance versus corresponding controls (unpaired Student’s t test) was as follows: **, p < 0.01; ***, p < 0.001; NS, not significant.
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response to B2 integrin engagement. We also found that pre-
treatment of PMNs with the NOS inhibitor L-NMMA (1 μM) totally blocked B2 integrin-induced accumulation of nitrite (Fig. 4C) thus confirming that nitrite produced by PMNs originate from NO.

Because Src tyrosine kinases, PI-3 kinase, PKC, and calcium mobilization were required for B2 integrin-induced activation of Rap1, and nitrite/NO is produced in response to B2 integrin engagement, we asked whether these signaling pathways were regulating the activity of NOS. To this end, we tested how inhibitors of these pathways affected B2 integrin-mediated activation of Rap1. As shown in Fig. 4D, engagement of B2 integrins on PMNs led to a 2.5-fold increase in nitrite production. However, pretreatment of PMNs with PP1 (3 μM), LY294002 (20 μM), GF-109203X (5 μM, GFX), or EGTA (1 mM) plus BAPTA-AM (30 μM, BAPTA) prior to engagement of B2 integrins caused a total block of nitrite production. Thus, Src tyrosine kinases, PI-3 kinase, PKC, and calcium signaling lie upstream of NOS, and these signaling molecules regulate NO production in human PMNs.

NO Is Involved in B2 Integrin-Induced Activation of Rap1 and Rap2—Because a stable end product of NO, nitrite, is produced in PMNs that have had their B2 integrins engaged, and NO donors have been shown to activate Rap1 in Jurkat T and PC12 cells (57), we next investigated whether NO was required for B2 integrin-induced loading of Rap1 and Rap2 with GTP. To this end, PMNs were pretreated with the NOS inhibitor L-NMMA (1 μM, 30 min), after which the cells were allowed to adhere to plates coated with fibrinogen in the presence of TNF (20 ng/ml, 20 min). Subsequently, the levels of GTP-bound Rap1 and Rap2 were determined as described above. We found that a pretreatment of PMNs with the broad range NOS inhibitor L-NMMA (1 μM) blocked significantly the B2 integrin-induced activation of Rap1 (Fig. 5A, left panel) and Rap2 (Fig. 5A, right panel). These results establish that NO is involved in B2 integrin-induced activation of Rap GTPases in these cells. Because PMNs express both iNOS (12) and neuronal NOS (13), we wanted to further characterize the nature of the NOS involved in the B2 integrin- and NO-dependent regulation of Rap GTPases. To this end, we used 1400W, a tight binding inhibitor of human iNOS (33). We found that a pretreatment of PMNs with 1400W (10 μM, 30 min) blocked B2 integrin-induced GTP loading of Rap1 (Fig. 5B, top panel) and Rap2 (Fig. 5B, bottom panel) similarly to the ability of the broad-range NOS inhibitor L-NMMA (1 μM, 30 min) to block Rap GTPase activation in response to B2 integrin engagement.

Rap1 has been shown to be a regulator of leukocyte adhesion through its ability to activate adhesion receptors such as B2 integrins (22, 58), and similarly to Rap1, Rap2 is a regulator of integrin-mediated adhesion of B cells (59). Because NO activates Rap1 and Rap2 in PMNs made adherent by B2 integrins, we assessed whether NO production was required for PMN adhesion. We found that adhesion of PMNs to a fibrinogen-coated surface, induced by TNF, was abolished if the leukocytes had been pretreated with L-NMMA (1 μM, 30 min) or 1400W (10 μM, 30 min) prior to their incubation on a plate coated with fibrinogen (Fig. 5C). Because PMN adhesion induced by TNF is largely dependent on augmented expression of B2 integrins on the cell surface (41), it is possible that L-NMMA impaired cell adhesion by blocking TNF-induced expression of B2 integrins on the cell surface. By flow cytometry, using a FITC-conjugated anti-human CD18 Ab, we have shown that treatment of suspended PMNs with TNF (20 ng/ml, 20 min) lead to an approximate 2-fold increase in the expression of CD18 on the cell surface (Fig. 5D). This is illustrated by the fact that the histo-
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FIGURE 5. NO is required for β2 integrin-induced activation of Rap1 and Rap2 and PMN adhesion.

A. Suspended PMNs (20 x 10⁶) were or were not pretreated for 30 min at 37 °C with the NOS inhibitor L-NMMA (1 μM). Non-adherent PMNs (treated with the vehicle) were taken as controls (time zero). Thereafter, the cells were incubated on a surface coated with fibrinogen in the presence of TNF (20 ng/ml) for 20 min after which the amounts of GTP-bound active Rap1 (left panel) and Rap2 (right panel) was measured. The inset show representative Western blots of each Rap GTPase. The diagrams illustrate densitometric analysis of the relative activities of Rap1 and Rap2 in PMNs that had been pretreated with L-NMMA versus suspended cells (time zero). B, suspended PMNs (20 x 10⁶) were or were not pretreated for 30 min at 37 °C with the NOS inhibitor L-NMMA (1 μM), or the specific NO synthase inhibitor 1400 W (10 μM). Non-adherent PMNs (treated with the vehicle) were taken as controls (time zero). Thereafter, the cells were incubated on a surface coated with fibrinogen and stimulated with TNF (20 ng/ml) for 20 min after which the amounts of GTP-bound active Rap1 (upper panel) and Rap2 (lower panel) was measured. The insets show representative Western blots of each Rap GTPase out of three experiments. C, PMNs (5 x 10⁶) were preincubated for 30 min at 37 °C with the fluorescent probe calcein AM (1 μM), after which L-NMMA (1 μM), 1400 W (10 μM), or the vehicle was added. The PMNs were then incubated on 96-well microplates coated with fibrinogen and stimulated with TNF (20 ng/ml) for 20 min. Non-adherent cells were subsequently removed by aspiration, after which fluorescence of each well was measured using a fluorescence microplate reader. Adhesion of PMNs was expressed as percentage of control cells (not stimulated with TNF, time zero) and represent means ± S.E. of four separate experiments. D, representative flow cytometric histograms depicting mean fluorescence intensity of CD18 expression on PMNs. PMNs were incubated with TNF, or pretreated with L-NMMA (1 μM), and incubated with TNF. Mouse IgG1-labeled cells were used as a negative control. Statistical significance versus corresponding controls (unpaired Student's t test) was as follows: * p < 0.05; ** p < 0.01; *** p < 0.001; NS, not significant.

to β2 integrin engagement and activation of iNOS, promote PMN adhesion via up-regulation of Rap1 and Rap2.

cGKI Is Involved in β2 Integrin-induced Activation of Rap GTPases and PMN Adhesion—PMNs express cGKI type I (19), therefore we assessed whether NO exerted its regulatory effect on Rap GTPases and PMN adhesion through activation of cGKI. To this end, we made use of Rp-8pCPT-cGMPS (cGKI I), which is an inhibitor of cGMP-dependent protein kinases 1α, 1β, and type II (35). Similarly to what we observed with L-NMMA, a pretreatment of PMNs with Rp-8pCPT-cGMPS (1 μM, 30 min) totally blocked β2 integrin-induced activation of Rap1 (Fig. 6A, left panel) and Rap2 (Fig. 6A, right panel), and PMN adhesion on fibrinogen in the presence of TNF (Fig. 6B). Furthermore, we also found that cGKI I did not affect the TNF-induced expression of β2 integrins on the cell surface (Fig. 6C). Thus, cGKI is involved in β2 integrin-induced NO-dependent activation of Rap GTPases and PMN adhesion.

Mn²⁺ Triggers a NO-dependent Activation of Rap GTPases—Similar to ligand mimetic peptides, Mn²⁺ induces a global conformational rearrangement in integrin extracellular domains that is linked to ligand binding (60). Mn²⁺ has been shown to induce a β2 integrin-dependent adhesion of human PMNs to fibrinogen as well as β2 integrin signaling (40). Therefore, to further confirm that β2 integrins were involved in the regulation of Rap GTPases in PMNs, we investigated whether stimulation of PMNs with the divalent cation would lead to activation of Rap GTPases. We found that stimulation of PMNs with Mn²⁺ (1 mM, 20 min) led to augmented levels of GTP-bound Rap1 (Fig. 7A, left panel) and GTP-bound Rap2 (Fig. 7A, right panel). Furthermore, pretreatment of PMNs with 1400 W (10 μM, 20 min) or cGKI I (1 μM, 20 min) resulted in a significant block of Mn²⁺-induced activation of Rap1 (Fig. 7A, left panel) and Rap2 (Fig. 7A, right panel). In parallel, Mn²⁺ promoted PMN adhesion on fibrinogen-coated plates as shown earlier by others (40), and this was blunted when PMNs had been pretreated with 1400 W (10 μM, 20 min) or cGKI I (1 μM,
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Figure 6. cGKI is involved in β2 integrin-induced activation of Rap1 and Rap2 and PMN adhesion. A, suspended PMNs (20 X 10^5) were or were not pretreated for 30 min at 37°C with the inhibitor of cGKI, R8-8pCPT-cGMPS (cGKI I, 1 μM). Non-adherent PMNs (treated with the vehicle) were considered as controls (time zero). Thereafter, the cells were incubated on a surface coated with fibrinogen in the presence of TNF (20 ng/ml) for 20 min after which the amounts of GTP-bound Rap1 (left panel) and Rap2 (right panel) was measured as described in the legend to Fig. 1. The insets show representative Western blots of each Rap GTPase. The densitometric analysis of the relative activities of Rap1 and Rap2 in PMNs that had been pretreated with R8-8pCPT-cGMPS versus suspended cells (time zero). B, PMNs (5 X 10^5) were preincubated for 30 min at 37°C with the fluorescent probe calcein AM (1 μM), after which R8-8pCPT-cGMPS (1 μM) or the vehicle was added. The PMNs were then incubated on 96-well microplates coated with fibrinogen and stimulated with TNF (20 ng/ml) for 20 min. Non-adherent cells were subsequently removed by aspiration, and the levels of adherent cells were determined as described in the legend to Fig. 5. Adhesion of PMNs was expressed as percentage of control cells (cells not stimulated with TNF, time zero) and represent means ± S.E. of five separate experiments. C, representative flow cytometric histograms depicting mean fluorescence intensity of CD18 expression on PMNs. PMNs were stimulated with TNF, or pretreated with R8-8pCPT-cGMPS (1 μM, cGKI I), and stimulated with TNF. Mouse IgG1, labeled cells were used as a negative control. Statistical significance versus corresponding controls (unpaired Student’s t test) was as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, not significant.

DISCUSSION

Other investigators (44) have studied regulation of Rap1 in human PMNs in response to ligands that act on G protein-coupled receptors, and they found that stimulation of PMNs with fMLP, platelet activating factor, or granulocyte macrophage-colony stimulating factor increases the levels of GTP-bound Rap1, but they failed to identify the signaling pathways downstream of the fMLP receptor that mediate activation of Rap1. Furthermore, in the cited study (44), no information was provided on the regulation of Rap2 in PMNs. Activation of Rap1 has also been observed in other cells of the immune system, where, for example, it is induced by B cell antigen (61) and T cell receptors (62). It is not yet known whether β2 integrins regulate Rap GTPases in leukocytes, therefore we investigated that possibility in human PMNs. Our results clearly establish that Rap1 and Rap2 are loaded with GTP upon engagement of the β2 integrins on PMNs that we induced either by incubating the cells in the presence of TNF or Mn^{2+} on a surface coated with fibrinogen (30, 40) or by plating the cells on a surface coated with an anti-CD18 antibody (29, 43).

A wide variety of inflammatory mediators induce functional activation of β2 integrins in leukocytes (inside-out signaling) (58), and there is growing evidence that Rap GTPases are involved in this process (22). Therefore, pro-inflammatory cytokines may regulate β2 integrins (and other integrins) in leukocytes, and thereby promote cell adhesion, by directly activating Rap GTPases. This appears to be the case for chemoattractants, such as fMLP, because stimulation of suspended PMNs with this compound induces activation of Rap1 (44) and enhances cell adhesion on a surface coated with ligands for β2 integrins (63). Accordingly, it was recently reported that chemokine-induced activation of Rap1 is abolished in leukocyte adhesion deficiency lymphocytes, and this event is associated with severe defects in integrin activation (64). In contrast, we found that the pro-inflammatory cytokine TNF did not induce significant activation of Rap1 or Rap2 in resting non-adherent PMNs. Therefore, we propose that the main role of TNF is to induce mobilization of β2 integrins to the cell surface, as suggested by others (41), whereas the basal levels of GTP-bound Rap1 and Rap2 are sufficient to induce a conformational change of β2 integrins necessary to initiate their ligand binding and signaling. Upon ligation of β2 integrins, Rap1 and Rap2 are further activated, and this will then in turn promote additional activation of integrins and augment PMN adhesion. In accordance, the induction of a conformational change of β2 integrins by incubating PMNs with Mn^{2+} triggers activation of Rap GTPases and increases the adhesion of PMNs to fibrinogen. The latter data are in good agreement with previously published results (40).

Activation of Src family tyrosine kinases is one of the earliest events in β2 integrin-mediated signaling, and it is essential for regulating the effects of β2 integrins on leukocyte functions (2). We found that β2 integrin-elicited stimulation of Rap1 requires Src tyrosine kinases but, surprisingly, those tyrosine kinases were not necessary for activation of Rap2. PI 3-kinase is another key downstream regulator of β2 integrin-induced modulation of functions in PMNs, and we noted that this lipid kinase was also involved, to some degree, in adhesion-dependent activation of Rap1 but not Rap2. Interestingly, our results suggest that...

20 min) (Fig. 7B). Thus, these results support the view that β2 integrin signaling (outside-in signaling) is responsible for the NO-dependent activation of Rap GTPases.
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PI 3-kinase and Src tyrosine kinases regulate Rap1 via a linear pathway, because the inhibitory effects of PP1 and LY294002 were not additive. This finding was anticipated because we have previously observed that, in human PMNs, PI 3-kinase is a downstream target of Src tyrosine kinases in the previously observed that, in human PMNs, PI 3-kinase is a pathway, because the inhibitory effects of PP1 and LY294002 were not additive. This finding was anticipated because we have previously observed that, in human PMNs, PI 3-kinase is a downstream target of Src tyrosine kinases in the β2 integrin signaling pathway (2, 65).

We also found that PKC and calcium signaling are involved in β2 integrin-induced activation of Rap1, and this could be readily ascribed to the fact that engagement of β2 integrins on PMNs activates PLCγ, an enzyme that catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate into 2 second messenger molecules: diacylglycerol (which activates PKC family members) and inositol trisphosphate (which induces release of calcium from intracellular stores) (7). Similarly, engagement of the B cell antigen receptor activates PLCγ and augments production of diacylglycerol, which is the second messenger that activates Rap1 (61). In platelets, ADP and thrombin-induced activation of Rap1 depend on elevation of the intracellular Ca2+ concentration (66), and the effect of ADP occurs through activation of Gq-coupled PLCβ.

Thus, we have identified some signaling molecules involved in the regulation of Rap1 by β2 integrins in human PMNs. Nevertheless, an unexpected discovery was that regulation of Rap2 by β2 integrins does not involve the Src tyrosine kinases, PI 3-kinase, PKC, or calcium signaling.

NO donors have been shown to augment the level of GTP-bound Rap1 in PC12 and Jurkat T cells (57), and, in human PMNs, expression of iNOS (which correlates with increased production of NO) is increased upon cell adhesion (15). Therefore, we next investigated whether iNOS, through production of NO, was involved in the β2 integrin-induced activation of Rap GTPases. We found that β2 integrin-dependent adhesion of PMNs led to accumulation of nitrite, a stable end product of NO. However, because nitrite production can also occur independently of NOS in PMNs (67), it was necessary to demonstrate that, in our experimental conditions, nitrite derived from NO. We obtained support for this notion by our observation that β2 integrin-induced accumulation of nitrite was totally blocked in PMNs pretreated with the broad range NOS inhibitor L-NMMA. We next established that NO plays an essential role in β2 integrin-induced activation of Rap GTPases in PMNs. This was demonstrated by showing that: 1) the β2 integrin-induced NO/nitrite production coincides with activation of Rap GTPases; 2) activation of Rap1 and Rap2 was

![FIGURE 7: NO is required for Mn2+–induced activation of Rap1 and Rap2 and PMN adhesion. A, suspended PMNs (20 × 10⁶) were or were not pretreated for 30 min at 37°C with the iNOS inhibitor 1400W (10 µM, W), or the cGKI inhibitor R-p-8pCPT-cGMPS (1 µM, cGKI). Non-adherent PMNs (treated with the vehicle) were taken as controls (time zero). Thereafter, the cells were incubated on a surface coated with fibrinogen and stimulated with MnCl₂ (1 mM, in the absence of TNF) for 20 min after which the amounts of GTP-bound Rap1 (left panel) and Rap2 (right panel) was measured. The insets show representative Western blots of each Rap GTPase out of three experiments. B, PMNs were pretreated or not with 1400W (10 µM, W), or the cGKI inhibitor R-p-8pCPT-cGMPS (1 µM, cGKI,), after which cells were incubated for 20 min on fibrinogen-coated plates in the presence of MnCl₂ (1 mM). Cell adhesion is then determined as described in the legend to Figs. 5 and 6. Statistical significance versus corresponding controls (unpaired Student’s t-test) was as follows: ***, p < 0.001; **, p < 0.01; *, p < 0.05; NS, not significant.](60x517 to 396x733)
occurred independently from these signaling molecules. Although, at present, we do not know all signaling pathways involved in β2 integrin regulation of Rap2, one possible and attractive explanation for our results could be that the activity of Rap2 is dually regulated by β2 integrins, in a positive and negative manner. In such a model, the NO- and cGMP-dependent activation of Rap2 is mediated via Src tyrosine kinases, PI 3-kinase, PKC, and calcium signaling. In addition, these signaling molecules might also be part of a NO- and cGMP-independent signaling pathway that regulates Rap2 in a negative manner. The additional actors involved in such a negative regulatory pathway of Rap2 remain, however, unknown. Hence, upon engagement of β2 integrins on PMNs, the basal activity of Rap2 is increased because the NO/cGMP pathway dominates over such an inhibitory pathway. However, when β2 integrin-induced activation of the NO/cGMP pathway is blocked by inhibition of Src tyrosine kinases, PI 3-kinase, PKC, or calcium signaling such an inhibitory pathway will also be blunted. This would lead to an increase in the basal Rap2 activity (see Fig. 8). We are presently investigating this hypothesis in our laboratory.

Very little information is available regarding the functional role of Rap GTPases in PMNs. Because Rap1 has been shown to be a regulator of cell adhesion in other leukocytes (22), and a similar function has been ascribed to Rap2 in B cells (59), we next investigated whether Rap GTPases were involved in PMN adhesion. We have provided evidence that this was the case. Indeed, a pretreatment of human PMNs with L-NMMA, 1400W, or Rp-8pCPT-cGMPS (which block the NO/cGMP pathway at different levels) blunted β2 integrin-induced activation of Rap1 and Rap2 and PMN adhesion on fibrinogen. Furthermore, we found that it was necessary to block Rap1 and Rap2 to prevent PMN adhesion by β2 integrins. This was illustrated by the fact that a pretreatment of PMNs with PP1, LY294002, GF109203X, or BAPTA-AM plus EGTA, which blocked β2 integrin-induced activation of Rap1, but not Rap2, failed to impair PMN adhesion on fibrinogen. Hence, this latter result might suggest that Rap2 but not Rap1 is critical for PMN adhesion.

Our results, showing that NO, generated in response to β2 integrin engagement, promotes PMN adhesion through activation of Rap GTPases, are in contrast to the work of other investigators, who have reported that exogenous NO, in the form of NO-releasing compounds, as well as cGMP analogues, inhibit adhesion of PMNs on vascular endothelial cells (69). This discrepancy may be explained by the fact that NO donors generate supra-physiological and inconsistent levels of NO, whereas, in our experimental conditions, physiological levels of NO are generated by PMNs. This idea agrees well with the notion that NO has a dual effect in PMNs, either blocking or activating PMN functions according to its local concentration (20).

In summary, we made several novel observations regarding regulation of Rap GTPases by β2 integrins in human PMNs. First, we found that ligation of β2 integrins on human PMNs activates Rap1 and Rap2. Second, we discovered that Rap1 and Rap2 are regulated by different signaling pathways that emanate from the β2 integrins. Third, engagement of β2 integrins on PMNs leads to production of NO, a gaseous signaling molecule that regulates Rap1 and Rap2 through activation of cGKI. Fourth, NO regulates PMN adhesion through its ability to activate Rap GTPases. Thus, inhibitors of the NO/cGMP pathway may be used as tools to block adhesion-induced activation of PMN functions by pro-inflammatory cytokines.
Acknowledgments—We thank J. L. Bos (University of Utrecht, The Netherlands) for his generous gift of the GST-RalGDS construct and Patty Odman for linguistic revision of the manuscript. We thank the blood donors and the staff at the Blood Centre of Malmö University Hospital (Malmö, Sweden) for kind and professional help. We also acknowledge Josie Scally, Morris McClelland (Blood Transfusion Centre, Belfast City Hospital), Eilish Armstrong and Hazel Johnston (Dept. of Geriatric Medicine, Queen’s University Belfast) for providing us with blood (ethical permission has been granted by the National Health Service Research Ethics Committee, Ref. 04/NIR01/78). We thank Adrien Kissennfjenn and Celine Gervin for performing flow cytometry experiments.

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