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# Characterization of Extracellular Nucleotide-Induced Mac-1 ($\alpha_M\beta_2$ Integrin) Surface Expression on Peripheral Blood Leukocytes

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**Extracellular nucleotides, released during vascular injury, stimulate hematopoietic cells resulting in various physiological responses. We have determined that nucleotides can stimulate the expression of Mac-1 on peripheral blood leukocytes. ATP stimulated the expression of Mac-1 in a time- and dose-dependent manner with maximum expression occurring in 5 min at 10 $\mu$M ATP. This increase in surface expression was observed in monocytes and granulocytes was dose-dependent and was comparable in extent to the increase induced by the chemotactic peptide, formyl-Met-Leu-Phe. Other nucleotides including 2-MeSADP, ADP, UTP, and 2MeSATP had similar effect. Nucleotide-mediated stimulation of Mac-1 expression in granulocytes was completely inhibited by Ro-31-8220, a specific inhibitor of protein kinase C, while variable inhibition was observed in monocytes. These results demonstrate the stimulation of peripheral blood leukocytes by nucleotides causing an increased surface expression of Mac-1 which may be mediated by the activation of protein kinase C.** 

Upon vascular injury, ATP and ADP are released in to the blood stream from damaged cells and from activated platelets and act on other blood cells, such as platelets, monocytes, lymphocytes, and neutrophils [1, 2]. In human neutrophils and the precursor cell line HL-60, ATP causes activation of phospholipase C (PLC) by both pertussis toxin sensitive and insensitive pathways [3]. Activation of PLC by ATP has been reported in macrophages [4]. Much less is known about the P2 purinoceptors in monocytes. Both ADP and ATP stimulate the phagocytic activity of neutrophils and monocytes [5] and increase intracellular calcium in monocytes and promonocytic U937 cells [2]. ADP also increases binding of fibrinogen [6] and coagulation factor X [7] to monocytes, and ATP enhances the binding of U937 cells to surfaces [8].

The effects of adenine nucleotides result from activation of cell membrane receptors, referred to as P2 purinoceptors [9]. P2 purinoceptors have been divided into two groups. Ligand-gated ion channels and non-specific pore receptors are designated P2X receptors [10]. The second group comprises of receptors coupled to G proteins. These include P2Y, P2U, P2T, and P2D subtypes [10]. All of these G protein-coupled receptors cause mobilization of intracellular calcium ions and/or activation or inhibition of adenylyl cyclase, and are designated P2Y receptors [10].

A variety of agonists, including the chemotactic peptide formyl-Met-Leu-Phe (fMLP), tumor necrosis factor, and platelet activating factor, stimulate the surface expression of the CD11b/CD18 integrin (Mac-1) on granulocytes [11]. This correlates with enhanced adherence of granulocytes to endothelial cells, adherence and spreading on artificial substrates, and granulocyte homotypic aggregation [11]. Granulocytes that lack Mac-1, from patients genetically deficient in Mac-1, show depressed baseline adherence. Similarly Monocytes show diminished adherence to endothelial cells and diminished chemotaxis [12]. Furthermore, a monoclonal antibody to the beta subunit of Mac-1 can block adherence by normal monocytes and granulocytes. These findings suggest that Mac-1 plays a role in the adherence of monocytes and granulocytes to endothelial cells [11, 12].

Mac-1 is also a receptor for C3bi and hence upregulation of Mac-1 may also enhance complement dependent monocyte effector functions [13]. When monocytes bind to fibronectin-coated surfaces C3bi-mediated phagocytosis is stimulated though the number of Mac-1 recep-

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Abbreviations: APCPP, $\alpha,\beta$-methylene ATP; APPCP, $\beta,\gamma$-methylene ATP; 2MeSADP, 2-methylthio ADP; 2MeSATP, 2-methylthio ATP; DMSO, Dimethyl sulfoxide; fMLP, formyl-Met-Leu-Phe.



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tors is unaffected [14]. Hence, increased surface expression of Mac-1 and monocyte binding to the extracellular matrix provide two independent mechanisms for enhancing monocyte complement receptor function during extravasation [15].

Mac-1 also plays an important role in blood coagulation by binding to fibrinogen [6], kininogens [16], and factor X [7]. Factor X binding sites on peripheral blood monocytes, identified as Mac-1, increase in response to ADP [7]. Thus Mac-1 may increase factor X concentration in the microenvironment and contribute to factor Xa formation and fibrin deposition at the site of vascular injury. Other components of the prothrombinase complex are also found on leukocyte surfaces [17, 18].

We have investigated the effect of extracellular nucleotides on the expression of Mac-1 on peripheral blood leukocytes.

## MATERIALS AND METHODS

*Materials.* Mouse IgG antibodies and monoclonal FITC-conjugated anti-human CD11b IgG mouse antibodies (product no. F-2648), and apyrase (Type VII) were purchased from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated mouse IgG antibodies were purchased from Becton-Dickinson. 4-$\alpha$ phorbol 12-myristate 13-acetate (4-$\alpha$-PMA), phorbol 12-myristate 13-acetate (PMA), and R0 31-8220 were purchased from LC laboratories (Woburn, MA).

*Isolation of blood cells.* Whole blood (50 ml/tube) was drawn from volunteers into citric acid-sodium citrate-dextrose, pH 6.4, and mixed with 0.1 volume of 7.5% polyanhydroglucose in polypropylene tubes and red blood cells were sedimented under gravity for 45 minutes at 4°C. Because Mac-1 expression in leukocytes can be induced by isolation procedures and by exposure to 37°C [15, 19], leukocytes were kept at 4°C during separation. Leukocyte-rich plasma was centrifuged at 400 X *g* for 30 minutes, and the pellet was resuspended in 5 ml of 155 mM ammonium chloride and 10 mM potassium bicarbonate, pH 7.4 to lyse the remaining red blood cells. To remove nucleotides released by damaged cells during the process, apyrase (final conc. 2.5 $\mu$g/ml) was included in the medium along with 1 mM magnesium chloride, and incubated at room temperature for 3-5 minutes. Leukocytes were washed twice in Hanks balanced salts solution containing 20 mM HEPES (HBSS) without $Ca^{+2}$ and $Mg^{+2}$ and the cell count was adjusted for each sample at 1 X $10^7$/ml.

*Flow cytometry.* These studies were carried out by standard procedures [20]. Isolated total blood leukocytes (1 X $10^6$/0.1 ml) were treated with 20 $\mu$l of mouse IgG (1 mg/ml) for 30 min at 4°C. The cells were then treated with agonist for specified time period at 37°C followed by 10 $\mu$l of FITC-CD11b antibodies at 4°C for 30 min. The cells were then fixed in 1% paraformaldehyde in Hanks balanced salt solution (HBSS). After washing twice they were stored in the dark until analysis in an Epics Elite flow cytometer (Coulter Corp., Hialeah, FL). For each experiment, cells treated with mouse IgG only, and cells treated with mouse IgG and mouse IgG-FITC were used as controls. The fluorescence of granulocytes, and monocytes were analyzed separately by gating on each respective cell population based on their different forward and side scattering patterns. At each point at least 5,000 cells were analyzed.

## RESULTS

*ATP-induced surface expression of Mac-1 on blood cells.* To avoid interference from nucleotides released from damaged cells during the isolation of leukocytes, we added apyrase to the wash buffers; the final suspension buffer did not contain apyrase. Cells were incubated with ATP (10 $\mu$M) or fMLP (100 nM) at 37°C for 10 min. Mac-1 expression was then assessed by flow cytometry using FITC labeled anti-CD11b antibody. Granulocytes and monocytes were resolved during fluorescence flow cytometry by gating on forward angle and side light scatter. The representative histograms are shown in Fig. 1A & B. fMLP, a known inducer of Mac-1 surface expression, enhanced Mac-1 expression ~4 fold on monocytes. ATP also caused ~4 fold increased surface expression of Mac-1 on monocytes. Parallel experiments on granulocytes also demonstrated ATP-induced Mac-1 surface expression.

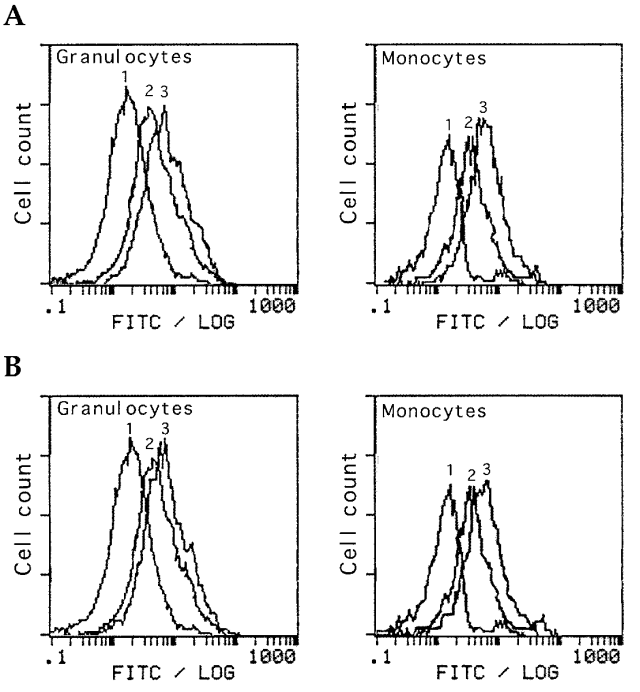


**FIG. 1.** Effect of ATP on Mac-1 surface expression on peripheral blood leukocytes. Aliquots of leukocytes isolated from blood collected in ACD were incubated at 37°C for 10 min with or without agonist as indicated. Cells were then labeled with FITC-anti-CD11b as described in the text and subjected to immunofluorescent flow cytometry. Monocyte and granulocyte populations were resolved by forward angle and 90° light scatter. Fluorescence histograms for each cell population were then collected and super-imposed. Panel A. ATP (10 $\mu$M) as agonist; Panel B, fMLP (100 nM) as agonist. Lines marked: 1, mouse IgG-FITC control; 2, no agonist control; and 3, agonist-stimulated.

*Time course of ATP-induced Mac-1 surface expression.* The large increase in Mac-1 expression within 10 min would appear to be too rapid for *de novo* protein synthesis. In order to determine the optimum incubation time for subsequent studies, leukocytes were incubated with fMLP (100 nM) or ATP (10 $\mu$M) for various time periods at 37°C and Mac-1 surface expression was

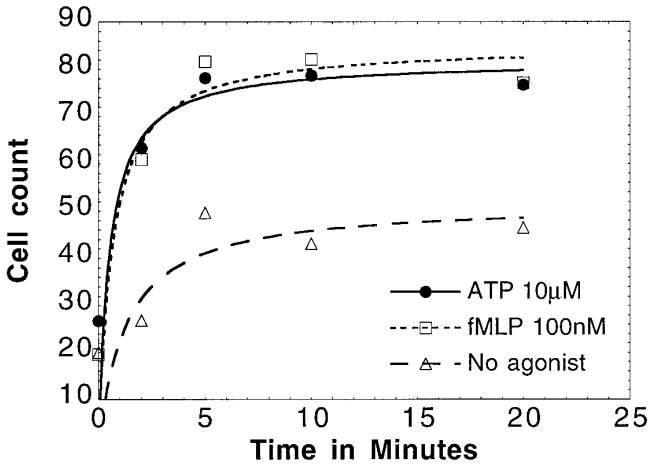


**FIG. 2.** Time course of Mac-1 surface expression. Peripheral blood monocytes were stimulated with ATP (10 $\mu$M) or fMLP (100 nM) for various time periods at 37°C and Mac-1 surface expression was determined.

measured. As shown in Fig. 2, Mac-1 appeared on monocyte cell surface within 2 min. and the surface expression was maximum at 5 min. Similar results were obtained for granulocytes (not shown).

*Dose-dependent expression of Mac-1.* The dose response curves for ATP-induced Mac-1 surface expression was determined and the results are shown in Fig. 3A. The upregulation of Mac-1 expression increased with increased concentration of ATP in monocytes and granulocytes. The expression was maximum on monocytes with an $EC_{50}$ of ~0.4 $\mu$M, followed by granulocytes with $EC_{50}$ of ~0.7 $\mu$M. The dose-dependent increases in Mac-1 surface expression were also seen with ADP (Fig. 3B) in monocytes and granulocytes, with $EC_{50}$ of ~0.3 $\mu$M, and ~0.15, respectively.

*Effect of ATP analogues on Mac-1 surface expression.* In order to determine the potency of other nucleotides on Mac-1 surface expression, several P2 purinoceptor agonists were used to stimulate leukocytes. As shown in Fig. 4, 2MeSADP, ADP, UTP, and 2MeSATP were highly effective with similar potency as ATP, while $\alpha,\beta$-MeATP, and $\beta,\gamma$-MeATP were less potent.

*Effect of R0-31-8220 (PKC inhibitor) on nucleotide-mediated Mac-1 surface expression.* Since all of the P2Y purinergic receptors so far cloned are known to activate phospholipase C, we determined the effect of a specific PKC inhibitor, Ro-31-8220, on nucleotide-mediated upregulation of Mac-1 expression. As shown in Fig. 5, R0-31-8220 completely inhibited ATP or ADP-mediated upregulation of Mac-1 surface expression in granulocytes, while ATP or ADP-induced Mac-1 expression on monocytes, was inhibited 70% and 35%, respectively.

## DISCUSSION

Two major sources of extracellular ATP in the blood stream are vascular injury, when the intact cells are mechanically broken and release cytosolic ATP, and stimulated exocytosis from the dense granules of platelets [1]. Extracellular nucleotides can act on a number of blood cells to trigger physiological responses. ATP stimulates phagocytosis in both neutrophils and monocytes. The importance of Mac-1 has been demonstrated in a number of granulocyte adhesion reactions [11].

We have demonstrated that extracellular ATP stimulates Mac-1 surface expression in monocytes and granulocytes. Stimulation was rapid, with maximum expression occurring within 10 min. The time course of stimulation was similar to the previously reported effect of fMLP [15, 21] and of ATP on granulocytes [21]. Such rapid expression is achieved by stimulation of transport of Mac-1 from intracellular vesicles to the surface, probably by vesicle fusion with the plasma

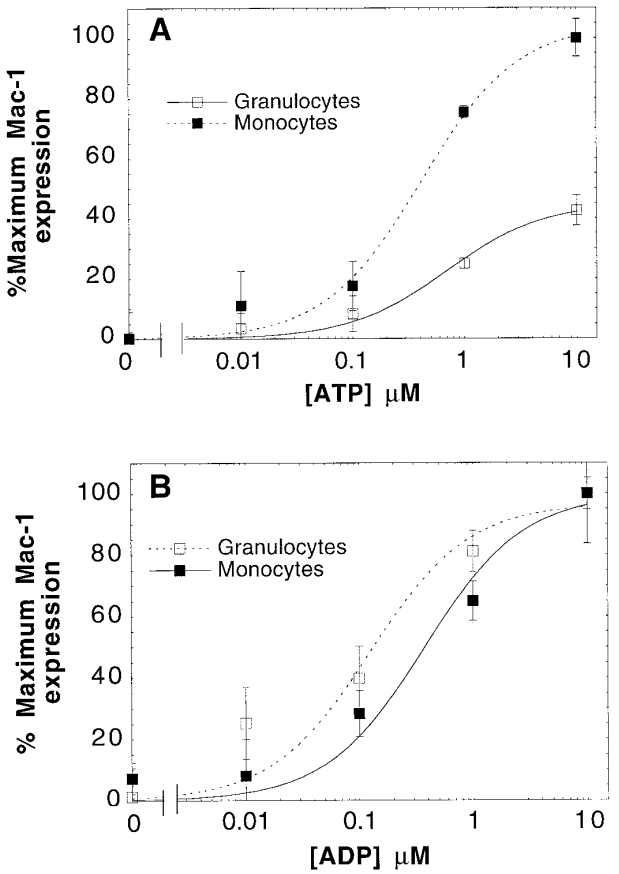


**FIG. 3.** Dose dependent surface expression of Mac-1 on peripheral blood leukocytes. Peripheral blood leukocytes were stimulated with varying concentrations of ATP (Panel A), or ADP (Panel B), for 5 min at 37°C and Mac-1 surface expression was determined as described in the text. The results were plotted after subtracting the Mac-1 expression without any agonist and normalizing to the maximum Mac-1 expression.

membrane [15]. The ATP-induced surface expression of Mac-1 was dose-dependent. Since the cell membrane is impermeable to ATP, the stimulation of Mac-1 surface expression must be a receptor-mediated event. Several nucleotide receptors have recently been cloned [reviewed in ref.22]. Several other nucleotides also stimulated Mac-1 surface expression and the potency of these nucleotides could mean several nucleotide receptors with different agonist profiles or a single nucleotide receptor with a unique agonist profile. While monocytes and granulocytes express the P2Y7 receptor (unpublished data from our laboratory), other P2 purinoceptors may also be present on these cells.

Phorbol esters can stimulate the expression of Mac-1 on the surface of neutrophils by stimulating PKC [23-25]. The $\beta$2 subunit (CD18) of Mac-1 is phosphorylated by PKC in vitro [26] and in vivo [27], and phorbol ester-stimulated aggregation of neutrophils is associated with increased phosphorylation of CD18. However, PKC does not appear to mediate the action of compliment component C5a, as Mac-1 up-regulation and increased endothelial cell adhesion stimulated by this agent are not inhibited by Ro-31-8425, a PKC specific inhibitor. It has been suggested that these responses to C5a are mediated through a protein kinase other than PKC, possibly a tyrosine kinase [25]. Extracellular adenine nucleotides have been shown to activate PKC in neutrophils [28]. In granulocytes, nucleotide-mediated up-regulation of Mac-1 expression was inhibited completely by a PKC specific inhibitor, Ro-31-8220 (Fig. 5) suggesting that the responses are mediated through activation of PKC.

Oxidized LDL increases Mac-1 surface expression on peripheral blood monocytes and promotes adhesion to endothelial cells through the activation of PKC [29]. On monocytes, the nucleotide-mediated response was only partly inhibited by Ro-31-8220, suggesting that pathways other than activation of PKC may also be involved in these cells. ADP and ATP activate distinct signal transduction pathways in promonocytic U937 cells [30]. It is possible that monocytes and granulocytes express different P2 purinoceptors and that Mac-1 up-regulation in these cells is mediated through multiple distinct pathways.

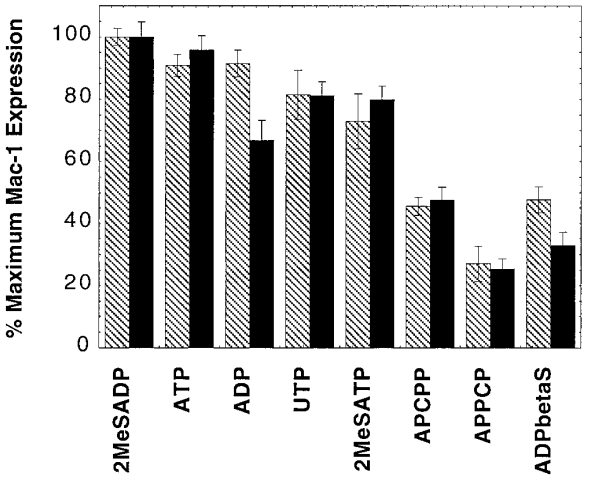


**FIG. 4.** Effect of ATP analogues on Mac-1 expression. Leukocytes were incubated with various ATP analogues (as indicated) at 37°C for 5 min and Mac-1 surface expression was determined. The data are plotted as percent maximum expression (obtained with 2MeS-ADP). The results were plotted after subtracting the Mac-1 expression without any agonist and are shown for monocytes (shaded bars) and granulocytes (filled bars).

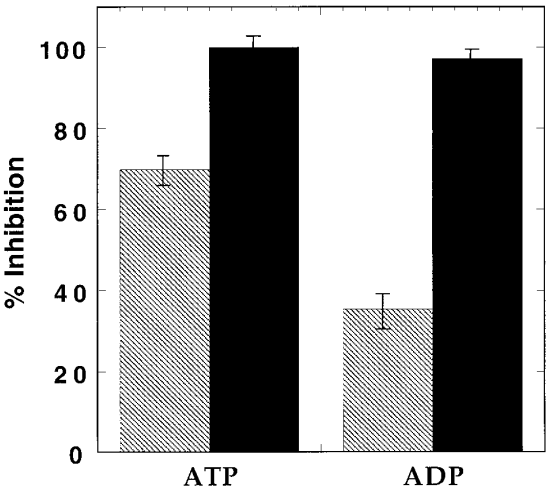


**FIG. 5.** Effect of Ro 31-8220 on nucleotide-induced Mac-1 expression. Peripheral blood leukocytes were incubated with Ro31-8220 or DMSO (buffer control) for 5 min prior to the addition of the agonists. The results were plotted after subtracting the Mac-1 expression obtained without any agonist and are shown for monocytes (shaded bars) and granulocytes (filled bars).

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