Journal of Leukocyte Biology 48:281–283 (1990)

SHORT COMMUNICATION

Effects of Adenosine on Inositol 1,4,5-Trisphosphate Formation and Intracellular Calcium Changes in Formyl-Met-Leu-Phe-Stimulated Human Neutrophils

Blair A.M. Walker, Brian E. Hagenlocker, Vonda K. Douglas, and Peter A. Ward

Department of Pathology, The University of Michigan Medicial School, Ann Arbor

In the presence of adenosine, formyl-Met-Leu-Phe-stimulated human neutrophils show a greatly diminished production of superoxide anion. Analysis of changes in levels of intracellular calcium revealed that the immediate increase (occurring within seconds) in intracellular calcium following addition of formyl-Met-Leu-Phe is not affected by the presence of adenosine, although there are significantly lower intracellular calcium levels during the late phase (occurring 1–4 min after addition of formyl-Met-Leu-Phe). Consistent with these findings is the fact that adenosine does not affect the production of inositol 1,4,5-trisphosphate in formyl-Met-Leu-Phe-stimulated neutrophils. These data suggest that the profound inhibitory effects of adenosine on superoxide responses in formyl-Met-Leu-Phe-stimulated neutrophils may be related to an action of adenosine occurring late in the sequence of events of signal transduction.

Key words: superoxide anion, phospholipase C, f-Met-Leu-Phe

Adenosine is known to be a powerful inhibitor of superoxide (O_2^-) and H_2O_2 responses in human neutrophils stimulated by formyl-Met-Leu-Phe (fMLP) [1–4,6,10,13,14]. Adenosine binds to high-affinity receptors on the human neutrophil, and, on the basis of varying inhibitory effects of different analogues of adenosine, the receptor falls into the A_2 class of adenosine receptors [4,9]. To complicate matters, adenosine does not inhibit human neutrophil chemotactic responses to fMLP [5,8], suggesting differences in the signal transduction pathways for chemotaxis and O_2^- production. The purpose of these studies was to evaluate in detail the ability of adenosine to affect fMLP-induced increases in intracellular calcium ($[Ca^{2+}]_i$) and production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. The data suggest that neither the generation of Ins(1,4,5)P_3 nor the immediate increase in $[Ca^{2+}]_i$ fMLP-stimulated neutrophils is affected by preincubation with adenosine.

Human peripheral blood neutrophils were isolated using Ficoll-Hypaque density gradient separation followed by lysis of red cells with ammonium chloride [10]. Superoxide formation was determined by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c [12]. When intracellular concentrations of calcium were assessed, cells were preloaded with fura-2/AM (Calbiochem, La Jolla, CA), and the resulting changes in fluorescence following cell stimulation with fMLP were measured in a Perkin-Elmer LS-5B luminescence spectrometer by continuous recording of intracellular fluorescence [11]. For convenience, the changes in $[Ca^{2+}]_i$ were calculated using calcium-dependent fura-2 fluorescent values obtained at quarter-or full-minute intervals. In appropriate experiments, 10 μM adenosine was added to the neutrophil preparation 3 or 5 min prior to addition of fMLP.

For the measurement of Ins(1,4,5)P_3, neutrophils were exposed to buffer or adenosine (10 μM) for 5 min followed by the addition of fMLP (100 nM). The reaction was terminated by the addition of an equal volume of ice-cold 20% trichloroacetic acid. The acid-insoluble material was removed by centrifugation, and the supernatant was extracted four times with water-saturated di-

Received March 22, 1990; accepted March 29, 1990.

Reprint requests: Blair A.M. Walker, Department of Pathology, The University of Michigan Medical School, 1301 Catherine St., Box 0602, Ann Arbor, MI 48109-0602.

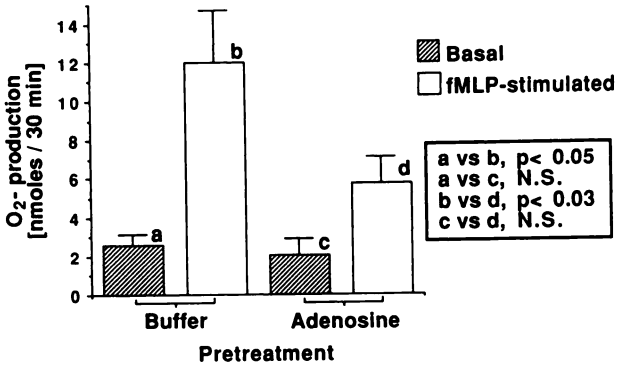

Fig. 1. Effects on O_2^- production in human neutrophils (2×10^6) stimulated with 500 nM fMLP in the presence or absence of 10 μM adenosine. Mean ± S.E. of four experiments. N.S., not significant.

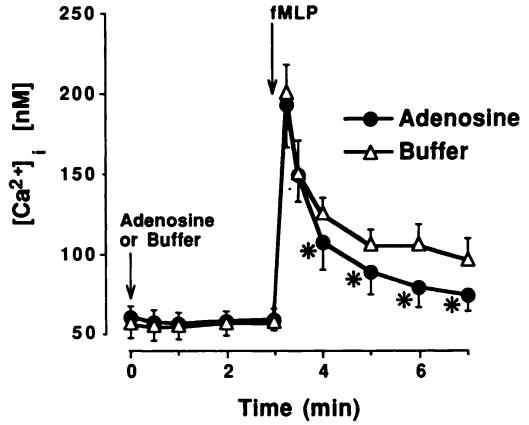

Fig. 2. Changes in intracellular calcium ($[Ca^{2+}]_i$, nM) in fura-2-loaded human neutrophils incubated with salt solution ("buffer") or with 10 μM adenosine followed by addition of 100 nM fMLP. *$P < 0.05$ comparing adenosine-treated to buffer-treated controls. Points represents mean ± S.E. of eight experiments.

ethyl ether and neutralized with 1 M $KHCO_3$. The Ins(1,4,5)P_3 mass was determined using a stereospecific binding assay [7] (Amersham International, Amersham, England). Ins(1,4,5)P_3 levels were measured at 0, 10, 20, and 30 sec following fMLP exposure. The highest Ins(1,4,5)P_3 mass was always measured at 10 sec. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. A paired Student's t test was used to compare all groups. Significance was defined as $P < 0.05$ and data are presented as mean ± S.E.

As has been documented elsewhere [1–4,6,10,13,14], 10 μM adenosine has a suppressive effect on the generation of O_2^- in 2×10^6 human neutrophils stimulated with 500 nM fMLP (Fig. 1). On the basis of four separate experiments, O_2^- production in unstimulated neutrophils was not affected by the presence of adenosine (approximately 2.5 nmole O_2^-/15 min; not significant), but there was a 54% suppression of O_2^- production in adenosine-treated neutrophils stimulated with fMLP.

As is shown in Figure 2, which represents the mean of eight separate experiments, the addition of adenosine (10 μM) did not alter $[Ca^{2+}]_i$ over the 3 min prior to the addition of fMLP. Exposure of adenosine-treated or buffer-treated neutrophils to fMLP (100 nM) results in a rapid rise in $[Ca^{2+}]_i$, which peaks within 15 sec. Adenosine pretreatment did not affect the peak $[Ca^{2+}]_i$ but did result in a more rapid fall in $[Ca^{2+}]_i$ that was significantly lower than in buffer-treated cells at 1, 2, 3, and 4 min after fMLP addition.

Figure 3 represents the mean of five separate experiments in which neutrophils were pretreated for 3 min with or without adenosine (10 μM) and then exposed to fMLP (100 nM). In the absence of adenosine, the Ins(1,4,5)P_3 mass in fMLP-stimulated neutrophils rose by 148%, to 5.13 ± 0.70 pmol/10^6 cells, whereas, in the

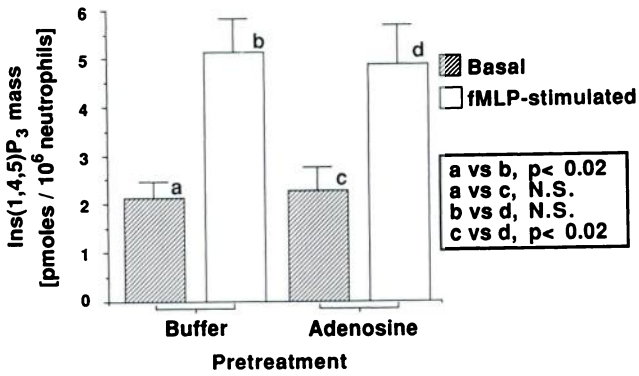

Fig. 3. Effects of adenosine on peak Ins(1,4,5)P_3 mass in extracts of neutrophils stimulated at 37°C with 100 nM fMLP. Peak Ins(1,4,5)P_3 mass were reached at 10 sec following the addition of fMLP. Mean ± S.E. of five experiments. N.S., not significant.

presence of adenosine, the rise was 115%, to a level of 4.87 ± 0.82 pmol/10^6 cells. There was no statistically significant difference between the peak Ins(1,4,5)P_3 responses in fMLP-stimulated neutrophils in the presence or absence of adenosine (Fig. 3).

The mechanism by which adenosine is able to inhibit O_2^- production in fMLP-stimulated neutrophils is not clearly established. The data in this report in which neither the generation of Ins(1,4,5)P_3 nor the immediate increase in $[Ca^{2+}]_i$ following stimulation of cells with fMLP is affected by the presence of adenosine suggests that the inhibitory capacity of adenosine may be at a step other than events leading to activation of phospholipase C. In other words, adenosine may not have as its target the early events in signal transduction.

In recent studies [10], we have demonstrated that hu-

man neutrophils exposed to adenosine show a slight reduction (10–18%) in the number of fMLP receptors, without a change in affinity. Since virtually identical changes in the number and affinity of fMLP receptors occur in cells exposed to adenosine-5′-O-(3-thiophosphate) (which enhances O_2^- responses to stimulation by fMLP), these observations suggest that the inhibitory effects of adenosine cannot be explained by the changes this purine compound induces in fMLP receptor content. These observations are consistent with the hypothesis that adenosine inhibits O_2^- production in fMLP-stimulated neutrophils via an effect on the late events in the signal transduction pathway. Indeed, although earlier studies had indicated that, with relatively high concentrations of the phorbol 12-myristate 13-acetate (PMA), adenosine has no inhibitory effect on O_2^- formation [2], we have recently been able to demonstrate that when the concentration of PMA is maintained at low levels (0.1–2.0 ng/ml), there is consistent and statistically significant inhibition (31–46%) of the O_2^- response in the presence of adenosine [10]. These data are consistent with concept that the inhibitory effects of adenosine might be directed at events related to activation of protein kinase C (e.g., proteolysis, translocation) or to a direct inhibitory effect on NADPH oxidase, although the latter seems less likely in that adenosine does not reduce the low level of O_2^- generation in "resting" neutrophils (Fig. 3).

ACKNOWLEDGMENTS

We acknowledge the contribution of Kimberly Drake in preparation of the manuscript. This work is supported by National Institute of Health grants HL31963, GM29507, and HL07517.

REFERENCES

1. Cronstein, B.N., Kramer, S.B., Rosenstein, E.D., Korchak, H.M., Weissmann, G., and Hirschhorn, R. Occupancy of adenosine receptor raises cyclic AMP alone and in synergy with occupancy of chemoattractant receptors and inhibits membrane depolarizations. Biochem. J. 252,709, 1988.
2. Cronstein, B.N., Kramer, S.B., Weissmann, G., and Hirschhorn, R. Adenosine: A physiological modulator of superoxide anion generation by human neutrophils. J. Exp. Med. 158,1160, 1983.
3. Cronstein, B.N., Kubersky, S.M., Weissmann, G., and Hirschhorn, R. Engagement of adenosine receptors inhibits hydrogen peroxide (H_2O_2) release by activated human neutrophils. Clin. Immunol. Immunopathol. 42,376, 1987.
4. Cronstein, B.N., Rosenstein, E.D., Kramer, S.B., Weissmann, G., and Hirschhorn, R. Adenosine. A physiologic modulator of superoxide anion generation by human neutrophils, Adenosine acts via a A2 receptor on human neutrophils. J. Immunol. 135,1366, 1985.
5. Garcia-Castro, I., Mato, J.M., Vasanthakumark, G. Weissmann, W.P., Schiffmann, E., and Chiany, P.K. Paradoxical effect of adenosine on neutrophil chemotaxis. J. Biol. Chem. 258, 4345, 1983.
6. Kubersky, S.M., Hirschhorn, R., Broekman, S.M., and Cronstein, B.N. Occupancy of adenosine receptors on human neutrophils inhibits the respiratory burst stimulated by the ingestion of complement coated particles and occupancy of chemoattractant but not Fc receptors. Inflammation 13,591, 1989.
7. Palmer, S., Hughes, K.T., Lee, D.Y., and Wakelam, M.J.O. Development of a novel, Ins(1,4,5)P_3-specific binding assay. Its use to determine the intracellular concentration of Ins(1,4,5)P_3 in unstimulated and vasopressin-stimulated rat hepatocytes. Cell. Signalling 1,147, 1989.
8. Rose, F.R., Hirschhorn, R., Weismann, G., and Cronstein, B.N. Adenosine promotes neutrophil chemotaxis. J. Exp. Med. 167,1186, 1988.
9. Schrier, D.J., and Imre, K.M. The effects of adenosine agonists on human neutrophil function. J. Immunol. 137,3284, 1986
10. Walker, B.A.M., Cunningham, T.W., Freyer, D.R., Todd, R.F. III, Johnson, K.J., and Ward, P.A. Regulation of superoxide responses of human neutrophils by adenine compounds. Independence of requirement for cytoplasmic granules. Lab. Invest. 61,515, 1989.
11. Ward, P.A., Cunningham, T.W., and Johnson, K.J. Signal transduction events in stimulated rat neutrophils: Effect of adenine nucleotides. Clin. Immunol. Immunopathol. 50,30, 1989.
12. Ward, P.A., Cunningham, T.W., McCulloch, K.K., and Johnson, K.J. Regulatory effects of adenosine and adenine nucleotides on oxygen radical responses of neutrophils. Lab. Invest. 58,438, 1988.
13. Ward, P.A., Cunningham, T.W., McCulloch, K.K., Phan, S.H., Powell, J., and Johnson, K.J. Platelet enhancement of O_2^- responses in stimulated human neutrophils: Identification of platelet factor as adenine nucleotides. Lab. Invest. 58,37, 1988.
14. Ward, P.A., Cunningham, T.W., Walker, B.A.M., and Johnson, K.J. Differing calcium requirements for regulatory effects of ATP, ATPγS and adenosine on O_2^- response of human neutrophils. Biochem. Biophys. Res. Commun. 154,746, 1988.