BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 209–212 (1997)
ARTICLE NO. RC976257

Evidence for the Activation of Myeloperoxidase by f-Meth-Leu-Phe Prior to Its Release from Neutrophil Granulocytes

Matthias Zipfel, Thomas C. Carmine, Claudia Gerber, Dietrich Niethammer, and Gernot Bruchelt[1]
Department of Hematology and Oncology, Children's University Hospital, D-72070 Tübingen, Germany

Received January 24, 1997

Activity and release of myeloperoxidase (MPO) was measured in heparinized whole blood samples after activation of neutrophil granulocytes by the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP) using two different methods: (i) by determination of the *amount* of MPO released into the blood plasma using a MPO enzyme-immunoassay, and (ii) simultaneously, by measuring the remaining *activity* within the neutrophils by flow cytometry using the Bayer Technicon H3. Although a part of MPO was released immedi ately after addition of fMLP, remaining MPO activity within the neutrophils surprisingly increased during the first minutes after incubation. Subsequently, MPO activity dropped due to a continuous release of MPO. In addition to fMLP, granulocyte-macrophage colony stimu lating factor (GM-CSF) enhanced MPO activity in neutrophils. These results indicate that MPO is present in resting granulocytes in an inactive or only partially active form and is activated by fMLP and GM-CSF. © 1997 Academic Press

Myeloperoxidase (MPO, E.C. 1.11.1.7) is produced by precursors of myeloid cells (mainly by myeloblasts and promyelocytes (1)) and is the most abundant enzyme in mature neutrophils (2). In cells activated e.g. by chemoattractants it transforms H_2O_2 generated during the oxidative burst in the presence of chloride to highly cytotoxic HOCl (3,4). MPO is stored in azurophilic granules from which it is partially released by activation with chemoattractants such as fMLP (5).

Measurement of peroxidase activity in individual leucocytes is the principle of white blood cell differentiation by the automatic hematological flow cytometry system Bayer Technicon H3 (6): After aspiration, whole blood is incubated with H_2O_2 and 4-chloro-1-naphthol in a H3-integrated reaction chamber leading to stained granulocytes and monocytes. Light scattering and absorbance are subsequently measured in single leucocytes by flow cytometry. In this way intracellular MPO *activity* of individual neutrophils is registrated. By investigating the blood of several thousands of healthy persons, mean MPO activity of the neutrophils was formerly estimated and expressed as mean peroxidase index (MPXI) which peaks at channel 28.5 on the x-axis in the leucogram (Fig. 1A). This value was arbitrarily set as zero, and a MPXI of 0 ± 10 is regarded as normal, comprising of about 90% of healthy population (6).

According to this principle, MPXI should drop if MPO is released from neutrophils, e.g. after incubation with fMLP (7). However, we surprisingly found, that immediately after incubation with fMLP MPXI first increased and only subsequently dropped due to a continuous release of MPO from the cells, indicating that MPO is activated by fMLP prior to its release from the granules.

MATERIALS AND METHODS

Blood samples. Heparinized whole blood samples of 9 male and female donors (21-46 years old) were used in this study. Granulocytes were isolated using a two step density gradient centrifugation using Histopaque 1077/1119 (Sigma, Deisenhofen, Germany).

Determination of intracellular MPO activity. The MPO activity of neutrophils was estimated in whole blood samples before and after incubation with the respective agents [Cytochalasin B, fMLP (Sigma), GM-CSF (Genzyme, Rüsselsheim, Germany)] by flow cytometry using the Bayer Technicon H3. As shown in Fig. 1A, software-controlled gating of the leucocytes places neutrophils right on the x-axis due to their high MPO activity, and MPXI is calculated for each neutrophil population (8). If the intracellular MPO activity decreases (e.g. by release of MPO), the neutrophils shift to the left which is associated with a fall of MPXI. Vice versa, if MPO activity increases, neutrophils shift to the right, and MPXI is enhanced.

Determination of MPO activity in extracts of isolated granulocytes. Isolated granulo cytes were adjusted at $4\text{-}6 \times 10^6$ cells/ml and incubated with 10^{-7} M fMLP in 10^{-5} M DMSO and 10^{-5} M DMSO (control) for 10 minutes at 37°C. Cell suspensions were subsequently sonified

[1] Corresponding author. Fax: ++49-7071-294448. E-mail: gernot.bruchelt@unituebingen.de.

0006-291X/97 $25.00

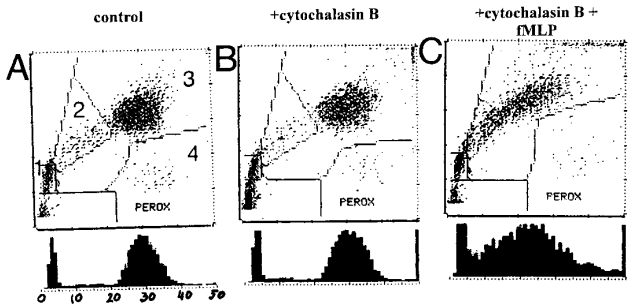


FIG. 1. Measurement of MPO release from neutrophils in whole blood samples by flow cytometry using the Bayer Technicon H3. (A) Characterisation of unstimulated white blood cells by measuring peroxidase activity (x-axis) and light scattering (y-axis). 1: Lymphocytes; 2: Monocytes; 3: Neutrophils; 4: Eosinophils. The higher the peroxidase activity of a single cell the more it is located towards the right on the x-axis. Below the graphs: Histograms of the peroxidase axis. (B) Same whole blood sample after treatment with cytochalasin B (5 μg/ml) for 10 minutes. (C) Sample B one minute after addition of fMLP (10^{-7} M).

and Triton X-100 (f.c.:0.1%) was added for total MPO release. 200 μl of cell extracts were incubated with 1.3 ml 2.5 mM 4-aminoantipyrine and 1.5 ml 1.7 mM H_2O_2 according to the procedure described in (9). Increase of absorption was measured at 510 nm.

Determination of MPO release. The amount of MPO released from neutrophils after treatment with the different stimuli was estimated using a MPO-enzyme-immunoassay (dis tributed by Biermann, Bad Nauheim, Germany): After distinct incubation times, whole blood was centrifuged and the blood plasma was frozen at −70°C until MPO analysis was carried out. In some experiments, released elastase was measured in parallel using an enzyme-immunoassay (Merck, Darmstadt, Germany).

RESULTS

Measurements of MPO release. The Bayer Technicon H3 measures intracellular MPO activity. Therefore, release of MPO should lead to a reduction of the mean peroxidase activity (MPXI). In order to clarify whether this process can be experimentally registrated, first experiments were carried in whole blood samples preincubated with cytochalasin B which enhances degranulation by fMLP due to its interaction with actin (10). Fig. 1C shows that an intense shift of the neutrophils to the left was achieved after addition of fMLP, indicating a strong reduction of intracellular MPO. In the example given, MPXI dropped from +4.6 to −10.8 after addition of fMLP (ΔMPXI = 15.4). Cells in the upper area of the graph (Fig. 1B and 1C) represent artefacts e.g. due to aggregations and are not taken into consideration by calculating the MPXI. In parallel, MPO release from neutro phils into the blood plasma was measured with an enzyme-immunoassay: The amount of MPO released arose from 11.5 ng/ml (untreated sample) up to 820 ng/ml (sample treated with cytochalasin B and fMLP).

The following experiments were carried out in the absence of cytochalasin B. Treat ment of whole blood samples with fMLP caused a rapid and time-dependent release of MPO as shown for three probands individually in Fig. 2A. Although the amount of MPO released into the blood plasma was distinctly smaller compared to samples pretreated with cytochalasin B, the effects could be easily registrated using the enzyme-immunoassay and correlated well with the amount of released elastase that is also stored in azurophilic granules (coefficient of regression: 0.97, data not shown). Based on these results it was ex pected that the intracellular MPO activity of the corresponding neutrophils (measured by flow cytometry) would be reduced. But, surprisingly, a significant increase of MPXI, i.e. an increase in intracellular MPO activity, was observed within the first minute after addi tion of fMLP, indicating that MPO was activated by fMLP prior to its release. Comparison of MPXI (Fig. 2B) with the corresponding amounts of released MPO (Fig. 2A) showed that MPXI even increased although part of the MPO was already released from the cells. Subsequently, MPXI was dropping due to the continuous release of MPO. Fig.3A sum marizes the results of another set of experiments showing the mean changes of MPXI 1,2,3,5 and 10 minutes after treatment of whole blood samples from six different donors with fMLP. The increase of MPXI during the first minute (ΔMPXI = 6.2 $\pm$ 2.0, n = 6) was significantly different from accidental fluctuations between individual measurements (ΔMPXI = 0.83 $\pm$ 0.36, n = 9). This was documented by repeated measurements of the MPXI of nine freshly isolated whole blood samples within a time period of 30 minutes, indicating only small variations of MPXI (Fig. 3B).

The results of the experiments carried out with whole blood samples were confirmed using isolated granulocytes: Pretreatment of cells with 10^{-7} M fMLP for 10 minutes enhanced MPO activity in one experiment by 25% and in another one by 36%, respectively (two different donors). These experiments were carried out by photometric measurements with extracts of pretreated and untreated granulocytes as described in Materials and Methods.

Influence of GM-CSF on intracellular MPO activity in neutrophils. Activation of MPO proved to be not restricted to pretreatment with fMLP. GM-CSF, which among other functions is known as a priming agent for neutrophils (11), was also able to activate MPO: Whole blood samples were incubated in the absence and presence of GM-CSF for 2 hours and MPXI values were registrated. As shown in Fig. 4A, all of the 7 different donors investigated showed a clear increase in MPO activity (Mean ΔMPXI = 4.75 $\pm$2.2, n=7). The histogram in Fig. 4B illustrates as an example the shift of MPO activity of neutrophils to the right after incubation with GM-CSF. Since mature neutrophils are not

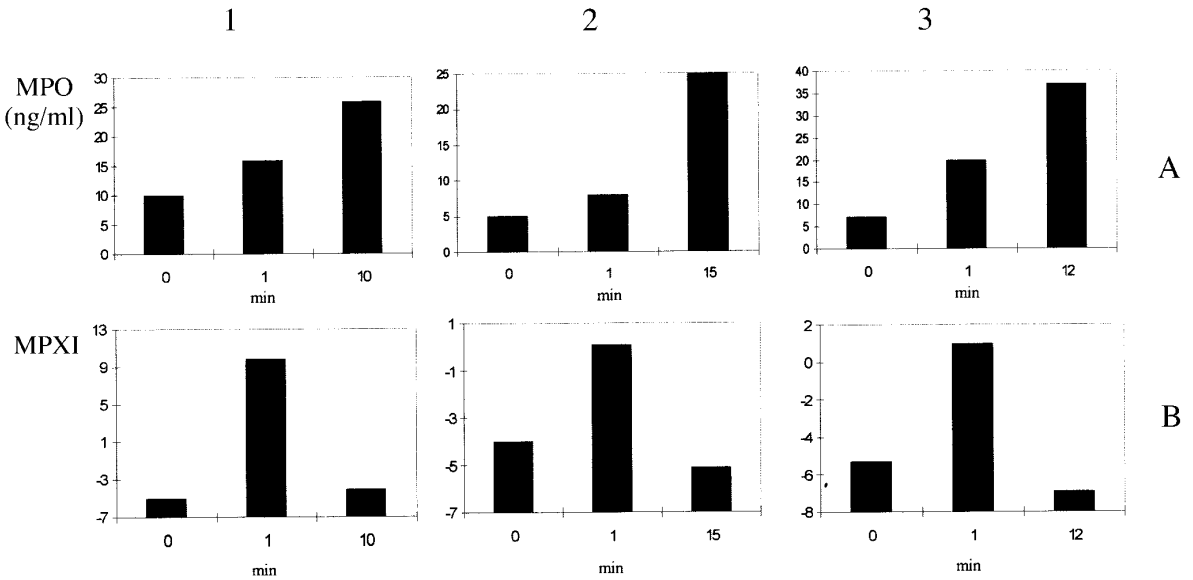


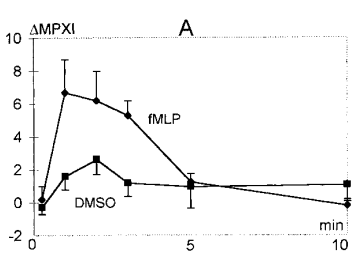
FIG. 2. (A) Time-dependent release of MPO from neutrophils of three donors after incubation of whole blood samples with 10^{-7} M fMLP (MPO mass released into blood plasma was measured by an enzyme-immunoassay). (B) Time-dependent changes of MPO activity (changes in MPXI) in the corresponding neutrophils of the same three donors after incubation with 10^{-7} M fMLP (whole blood measurements on the Bayer-Technicon H3).

able to syn thesize MPO (12) these data indicate that GM-CSF, like fMLP, also enhances MPO activity.

DISCUSSION

It was reported that MPO activity could be modulated by different substances, e.g. by superoxide anion (13,14). Data of the present study indicate that MPO in neutrophils is activated by fMLP and GM-CSF. Neutrophils of all donors investigated in this study showed a significant increase in MPO activity after treatment with both substances. Using the H3 flow cytometry system, MPO activity was determined in intact neutrophils of whole blood samples immediately after blood drawing, i.e. under almost physiological conditions. In this way potential unspecific activation of the fragile granulocytes, e.g. due to mechanical stress during the isolation procedure, can be largely prevented. Nevertheless, higher MPO activities were also observed in extracts of isolated granulocytes after pretreatment with fMLP compared to untreated controls.

The experiments shown in this paper indicate that MPO is present in resting neutrophils in an only partially activated state or, perhaps, even in an inactive form. HOCl generated in the MPO reaction in the presence of chloride and H_2O_2 is a highly aggressive substance (15), and, therefore, it is advantageous for resting granulocytes to harbour MPO in an in active state. Deleterious effects by accidentally generated H_2O_2 can be easier prevented since hydrogen peroxide is much



B

Donor	MPXI	n
1	-8,35±0,64	4
2	-7,80±1,15	4
3	-8,60±0,36	6
4	-4,90±1,19	9
5	6,50±0,91	3
6	-7,00±0,80	8
7	8,30±1,40	6
8	-8,40±0,37	8
9	-8,70±0,67	8

n=number of measurements within 30 minutes
(mean +S.D.)

FIG. 3. (A) Mean changes of MPXI after incubation of whole blood from 6 donors with 10^{-7} M fMLP during the first 10 minutes after its application. As a control, the influence of DMSO (soluent for fMLP) was registrated. Mean ± S.D. (B) Fluctuation of MPXI in whole blood samples from 9 donors measured several times during a period of 30 minutes.

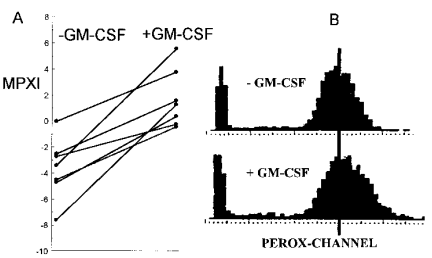


FIG. 4. (A) Increase of MPO activity (MPXI) in neutrophils of 7 different donors after incubation of whole blood samples with 500 U/ml GM-CSF for 2 hours compared to un treated controls. (B) Histogram of MPO activity in the neutrophils of one donor in the absence (MPXI = −2.5) and presence (MPXI = +1.6) of GM-CSF. (The vertical line drawn at channel 30 is included for an easier registration of granulocyte shift).

less aggressive than HOCl, and more effective intracellular scavenger systems exist for H_2O_2 than for HOCl. Neutrophils contain other enzymes, such as collagenase and gelatinase, that are operative only after stimulation. Interestingly enough, collagenase as well as gelatinase can be activated by HOCl (16). Therefore, also from this point of view it is advantageous for the resting granulocyte to contain MPO in an inactive or only partially active state in order to prevent self destruction or damage of surrounding tissue.

REFERENCES

1. Tobler, A., Miller, C. W., Johnson, K. R., Selsted, M. E., Rovera, G., and Koeffler, H. P. (1988) *J. Cell. Physiol.* **136,** 215–225.
2. Nauseef, W. M., McCormick, S., and Yi, H. (1992) *Blood* **82,** 1317–1322.
3. Raggiolini, M., Boulay, F., Badwey, J. A., and Curnutte, J. T. (1993) *FASEB J.* **7,** 1004–1010.
4. Klebanoff, S. J. (1970) *Science* **169,** 1095–1097.
5. Schiffman, E., Aswanikumar, S., Venkatasubramanian, K., Corcoran, B. A., Pert, C. B., Brown, J., Gross, E., Day, A. R., Freer, R. J., Showell, A. H., and Becker, E. L. (1980) *FEBS Letters* **117,** 1–7.
6. Kutter, D., Al Haidari, K., and Thoma, J. (1994) *Klin. Lab.* **40,** 337–341.
7. Lippi, U., Bellavite, P., Schinella, M., Nicoli, M., and Lippi, G. (1994) *Eur. J. Clin. Chem. Clin. Biochem.* **32,** 11–17.
8. Kutter, D., Hendriks, J. Ph., Missenard, C., Musilova, L., and Winter, F. (1994) *GIT Labor-Medizin* **17,** 68–72.
9. Metcalf, J. A., Gallin, J. I., Nauseef, W. M., and Root, R. K. (1986) Laboratory Manual of Neutrophil Function, Raven Press, New York.
10. Rosen, G. M., Pou, S., Ramos, C. L., Cohen, M. S., and Britigan, B. E. (1995) *FASEB J.* **9,** 200–209.
11. Weisbart, R. H., Golde, D. W., Clark, S. C., Wong, G. C., and Gasson, J. C. (1985) *Nature* **314,** 361–363.
12. Richter, J., Anderson, T., and Olsson, I. (1989) *J. Immunol.* **42,** 3199–3205.
13. Hurst, J. K., and Barrette, W. C., Jr. (1989) *Crit. Rev. Bioch. Mol. Biol.* **24,** 271–328.
14. Kettle, A. J., and Winterbourn, C. C. (1988) *Biochem. J.* **252,** 529–536.
15. Weiss, S. J., Peppin, G., Ortiz, X., Ragsdale, C., and Test, S. T. (1985) *Science* **227,** 747–749.
16. Weiss, S. J. (1989) *N. Engl. J. Med.* **320,** 365–376.