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Intracellular Reactions in Single Human Granulocytes upon Phorbol Myristate Acetate Activation using Confocal Raman Microspectroscopy

Nanna M. Sijtsema,* Arjan G. J. Tibbe*, Ine G. M. J. Segers-Nolten*, Arthur J. Verhoeven,† Ron S. Weening,‡ Jan Greve,* and Cees Otto*

*University of Twente, Institute for Biomedical Technology, Department of Applied Physics, Applied Optics Group (TOP), 7500 AE Enschede; †Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, 1006 AD Amsterdam; and ‡Emma’s Children’s Hospital, Academic Medical Center, Amsterdam, the Netherlands

ABSTRACT We have obtained new evidence for the occurrence of intracellular NADPH-oxidase activity in neutrophilic and eosinophilic granulocytes upon stimulation with phorbol myristate acetate (PMA). PMA activation leads to a partial translocation of cytochrome \( b_{558} \) from the membranes of the specific granules to the plasma membrane. It was suggested that NADPH-oxidase activity only takes place in the plasma membrane, leading to an extracellular release of oxygen metabolites because cellular self-destruction can be avoided in this way. The effects of PMA activation were indirectly studied in recent experiments employing scavengers of extracellular superoxide anion and hydrogen peroxide, and support for intracellular NADPH-oxidase activity was obtained. In this paper we use Raman microspectroscopy as a direct method to study intracellular molecular reactions that result from cellular triggering by PMA. The molecular specificity of this microscopic method enables us to show that intracellular reduction of both myeloperoxidase (MPO) and cytochrome \( b_{558} \) occurs in neutrophilic granulocytes. Control measurements with cytochrome \( b_{558} \)-deficient neutrophilic granulocytes did not show a reduction of intracellular MPO. This is direct support for the occurrence of intracellular NADPH-oxidase activity in organelles that must be in close contact with the azurophilic granules that contain MPO. Furthermore, a comparison was made with chemical reactions occurring in eosinophilic granulocytes after activation with PMA. Moreover, in these cells an intracellular reduction of eosinophil peroxidase was observed.

INTRODUCTION

Neutrophilic granulocytes play an important role in host defense against invading organisms (Borregaard, 1988). Among the proteins that are of importance in this process are myeloperoxidase (MPO), concentrated in the azurophil or primary granules, and the membrane-bound cytochrome \( b_{558} \) (also known as flavocytochrome \( b \) or cytochrome \( b_{245} \)), concentrated in the specific or secondary granules and present in the secretory granules. Cytochrome \( b_{558} \) is the central protein in the phagocytic NADPH-oxidase system of neutrophilic and eosinophilic granulocytes and other phagocytes, which converts oxygen into superoxide anion (\( O_2^- \)). Furthermore, at least three different cytosolic components (Clark et al., 1990; Knaus et al., 1991) are necessary for this reaction. Cytochrome \( b_{558} \) consists of two membrane-bound redox centers. One center is a FAD-containing flavoprotein. The other center contains a heme group as well as the NADPH binding site (Segal et al., 1992; Rotrosen et al., 1992; Sumimoto et al., 1992). It is assumed that there is an electron flow from NADPH to oxygen (Cross and Curnutte, 1995), leading to the formation of superoxide anions. These superoxide anions dismutate spontaneously, and \( H_2O_2 \) is produced quantitatively (Test and Weiss, 1984). MPO reacts with this \( H_2O_2 \) to produce the short-lived catalytic intermediate compound I (with an oxidation state of two oxidation equivalents above that of the resting native enzyme), which reacts with chloride to form the bactericidal agent hypochlorous acid (HOCl) (Klebanoff, 1991):

\[
MPO + H_2O_2 \quad \rightarrow \quad \text{Compound I} + H_2O
\]

\[
\text{Compound I} + H^+ + Cl^- \quad \rightarrow \quad \text{MPO} + HOCl
\]

Neutrophils from people who suffer from the X-linked form of chronic granulomatous disease (CGD) lack cytochrome \( b_{558} \) and are incapable of generating superoxide anions, which results in severe, recurrent infections (Baehner and Nathan, 1967; Curnutte et al., 1974; Smith and Curnutte, 1991). People with a partial or total MPO deficiency do not have these problems, although candidiasis, a fungal infection, has been reported to occur frequently in people with such a deficiency (Nauseef, 1990; Lehrer and Cline, 1969). These clinical data indicate that cytochrome \( b_{558} \), in contrast to MPO, is required for the killing of invading microbes.

Eosinophilic granulocytes can also form the bactericidal agent hypochlorous acid in a process similar to that in neutrophilic granulocytes, but with a lower efficiency (Klebanoff et al., 1989; Henderson, 1991). Eosinophils use eosinophil peroxidase (EPO) instead of MPO as the redox center (Wever and Plat, 1981). Eosinophils and neutrophils can be activated in vitro by the soluble activator phorbol.
myristate acetate (PMA), which activates NADPH oxidase (Majumdar et al., 1991; Dusi and Rossi, 1993). It is not clear whether this NADPH oxidase activity only takes place at the plasma membrane, as is suggested in many publications (Babior, 1984; Rossi, 1986; Bellavite, 1988), or can also occur intracellularly. Recent experiments showed that scavengers of extracellular \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) hardly affected the PMA-induced chemiluminescence response in human neutrophils, indicating an intracellular localization of the activity (Vaisiere et al., 1995; Lundqvist et al., 1996). Absorption measurements on highly concentrated PMA-activated neutrophilic granulocytes in suspension showed a reduction of cytochrome \( b_{558} \) under anaerobic conditions (Cross et al., 1982; Iizuka et al., 1985) and the formation of MPO, other redox states of MPO and cytochrome Raman Spectroscopy in Activated Granulocytes 2607

In the present paper we use resonant confocal Raman microspectroscopy to study the changes in the cytoplasm of the redox state of MPO and cytochrome \( b_{558} \) in neutrophilic granulocytes and EPO in eosinophilic granulocytes after activation with PMA. In particular, we have obtained direct evidence for intracellular NADPH oxidase activity after PMA activation. Resonant Raman spectra of MPO- and cytochrome \( b_{558} \)-deficient granulocytes with reduced redox centers allowed a complete interpretation of the spectral changes.

**EXPERIMENTAL PROCEDURES**

For the Raman measurements neutrophilic granulocytes from the peripheral blood were isolated from fresh heparinized blood as described previously (Yazdanbakhsh et al., 1987). The neutrophile granulocytes from the CGD donor were isolated in the same way. The neutrophils from the CGD donor are characterized as follows: no cytochrome \( b_{558} \) subunits detectable on a Western blot, a deletion of two nucleotides in exon 7 of the gene encoding for gp91-phox (AA on position 752/753), and an insertion of a T at this position. Quartz plates were incubated overnight with 0.01% PLL (poly-L-lysine, P-1274; Sigma, St. Louis, MO) in phosphate-buffered saline at 4°C. A few drops of \( (2 \times 10^6) \) cells/ml suspended in medium 1 (RPMI 1640 + 25 mM HEPES without phenol red (Seromed, Berlin, Germany) with 3% fetal calf serum (011-06180; Gibco, Paisley, Scotland)) were put on a PLL-coated quartz glass. After an incubation of 10 min at 37°C the quartz glass was put in a Petri dish (35 mm), and 2 ml of medium 1 was added. During the measurements the sample was kept at 37°C.

For the absorption measurements neutrophile granulocytes from the peripheral blood were isolated from buffy coats from the Central Blood Bank (Enschede, the Netherlands) (Yazdanbakhsh et al., 1987). Cells (2.5 ml of \( 5 \times 10^7 \) cells/ml in medium 1) were put in a quartz cuvette (Hellma Standard Cuvet 110QS). Absorption measurements were performed on a Shimadzu UV-2101PC spectrophotometer with an integrating sphere to diminish the effect of scattering. Absorption difference spectra are presented as \( \Delta (\text{sample}) - \Delta (\text{suspension of native neutrophils}) \).

In both the Raman and the absorption measurements the neutrophils were activated by the addition of 0.1 \( \mu \text{g/ml} \) PMA (phorbol-12-myristate-13-acetate, P-8139; Sigma). Complete reduction of the redox centers inside the neutrophils was achieved by the addition of sodium dithionite.

A confocal Raman microspectrometer (Sijtsema et al., 1998b) was used to measure the Raman spectra. The 413.1-nm line of a krypton laser (Innova 90-K; Coherent, Santa Clara, CA) is reflected by a beamsplitter (bs) (reflection 30%, transmission 70% for 413 nm) and is focused onto the sample by a microscope objective (Zeiss Plan Neofluar, 63×, numerical aperture 1.2, water immersion; Carl Zeiss, Jena, Germany). The scattered light is collected by the same objective and transmitted through the beamsplitter. A holographic notch filter (nf) (Kaiser Optical Systems, Ann Arbor, MI) is used to suppress reflected laserlight and Rayleigh scattered light. The scattered light is focused onto a pinhole (50 \( \mu \text{m} \)) positioned at the entrance of a Jobin-Yvon HR460 imaging spectrograph/monochromator (ISA; Jobin-Yvon, Paris, France) containing a blazed holographic grating with 1200 gr/mm (630-nm blaze). A Princeton liquid nitrogen-cooled CCD detector containing a back-illuminated chip with 1100 × 330 pixels of \( 24 \times 24 \mu \text{m}^2 \) (LN/CCD 1100 PB/VisAR; Princeton Instruments, Trenton, NJ) is placed in the focal plane of the spectrograph exit port and is used to measure the Raman spectra of a small sample volume.

The spatial resolution (full width at half-maximum) of the set-up was determined to be 0.37 \( \mu \text{m} \) in the lateral direction and 1.2 \( \mu \text{m} \) in the axial direction for a small sphere (diameter 0.282 \( \mu \text{m} \)) and 3.6 \( \mu \text{m} \) in the axial direction for a thin layer (thickness 0.28 \( \mu \text{m} \)) (Sijtsema et al., 1998b). The axial resolution for cellular samples will be between 1.2 and 3.6 \( \mu \text{m} \). The spectral resolution was \( \approx 4 \text{ cm}^{-1} \). The set-up is integrated with a bright-light microscope. A bright-light image of the cells together with a weak (nanoW) laser spot can be monitored during alignment. The sample was positioned with the laser spot coinciding with the area of interest in the selected cell.

The spectra of neutrophile and eosinophilic granulocytes were measured after focusing the laser beam (wavelength 413.1 nm) in a spot with a large concentration of granules inside the cell. These spots with high granule concentrations could easily be recognized in the bright-light image of the cells. The Raman signal of the heme groups in cytochrome \( b_{558} \) and...
EPO is resonantly enhanced because the excitation wavelength corresponds to the Soret band in the absorption spectra of these compounds. A smaller resonant enhancement of the Raman signal of the heme group in MPO is expected because of its redshifted Soret band ($\lambda_{\text{max}} = 430$ nm) (Kooter et al., 1997). A decrease in the Raman signal of MPO, EPO, and cytochrome b$_{558}$ was observed during the illumination of the sample. This bleaching is probably caused by photodestruction of the proteins. We have avoided the effect of bleaching in our measurements by optimizing the measurement time and the laser power. With a laser power of 0.5 mW on the sample and a measurement time of less than 15 s, no decrease in the Raman signal could be detected. We proceeded to measure every cell only once over 10 s with a laser power of 0.5 mW. The white-light microscope option integrated with the Raman microspectroscope was used to recognize flat cells that had adhered to the quartz glass. These cells were not measured.

Difference spectra were calculated by subtracting spectra of the nonactivated eosinophils and neutrophils from spectra of the PMA-activated or dithionite-reduced cells. Before the difference spectra were calculated, the spectra of nonactivated neutrophils and eosinophils were averaged over −15 measurements, filtered with a fast Fourier transform filter, and scaled on the 677 cm$^{-1}$ band of the activated cell spectra. This scaling was necessary to correct for the differences in MPO or cytochrome b$_{558}$ concentrations in the measurement volume between different cells. Scaling on the 677 cm$^{-1}$ band can correct for this effect, because the Raman spectra (with 413-nm excitation) of isolated MPO and cytochrome b$_{558}$ show that the intensity of this band is hardly influenced by a reduction of the proteins.

RESULTS AND DISCUSSION

Absorption spectra for a suspension of highly concentrated neutrophilic granulocytes were measured at various times after activation with PMA. In Fig. 1 are presented absorption difference spectra for (Fig. 1 A) sodium dithionite-reduced and PMA-activated neutrophils measured (Fig. 1 B) 11 min and (Fig. 1 C) 3 min after the addition of PMA. Fig. 1, A and B, shows a distinct shift in the Soret maximum of cytochrome b$_{558}$ from 410 nm to 428 nm. In the Q-band region an increase in absorption can be observed around 560 nm and a decrease can be observed at 577 nm, which are also due to a reduction of cytochrome b$_{558}$. The band at 470 nm indicates a reduction of MPO. About 10% of the MPO and 40% of the cytochrome b$_{558}$ were in the reduced state ~11 min after the addition of PMA, if we assume a 100% reduction of both proteins in Fig. 1 A. Within 3 min after the addition of PMA (Fig. 1 C) no reaction could yet be observed.

Confocal Raman microspectra of single neutrophilic granulocytes were measured. The (confocal) resolution, together with the molecular specificity of this technique, is used to determine whether the chemical reactions we observe in the absorption measurements occurring intracellularly or extracellularly. In Fig. 2 a representative set of single-cell Raman difference spectra (see Materials and Methods) is shown. The Raman difference spectra were obtained 32 (Fig. 2 A), 28 (Fig. 2 B), 16 (Fig. 2 C), and 14 min (Fig. 2 D) after the addition of PMA. Only ~10 min after the addition of PMA a reaction could be observed in the cells. The magnitude of the spectral changes increased as a function of time until ~20–30 min after the addition of PMA and varied from cell to cell. The spectral changes presented in Fig. 2 can be completely interpreted in terms of changes in both MPO and cytochrome b$_{558}$ (Sijtsema et al., 1998). The magnitude of the contribution of each of these compounds can be obtained from a comparison of the Raman spectra of neutrophilic granulocytes from healthy donors with those from a MPO-deficient donor and those from a CGD patient (see Materials and Methods). In Fig. 3 the Raman difference spectra (reduced-oxidized) for normal (Fig. 3 A), MPO-deficient (Fig. 3 B), and CGD neutrophils (Fig. 3 C) are presented. The difference spectra were obtained by subtracting the Raman spectrum of the native cells (with oxidized redox centers) from that of sodium dithionite-treated cells (with reduced redox centers). A positive band at 1528 cm$^{-1}$ and a negative band at 1580 cm$^{-1}$ are specific for the reduction of cytochrome b$_{558}$, whereas positive bands at 1473 and 1606 cm$^{-1}$ and negative bands at 1553, 1597, and 1615 cm$^{-1}$ are specific for a reduction of MPO. In Table 1 an overview of the positions of the most important Raman bands measured in normal, MPO-deficient, and CGD neutrophils is given.

In Fig. 3 the results on PMA-activated cells from a normal donor (Fig. 3 D), a MPO-deficient donor (Fig. 3 E), and a cytochrome b$_{558}$-deficient chronic granulomatosus disease (CGD) patient (Fig. 3 F) are given. The result in Fig.
cytochrome
neutrophils (Fig. 3
different donors. The difference spectrum of MPO-deficient
normal neutrophils isolated on different days from two
upon PMA activation were observed in four batches of
obtained after the addition of dithionite). The same reactions
state after activation with PMA (a 100% change being
6
3
3
and 30
2
2
cm
2

FIGURE 2 Raman difference spectra of single PMA-activated neutro-
phils measured at (A) 32, (B) 28, (C) 16, and (D) 14 min after the addition
of PMA. Fourteen minutes after the addition of PMA a reaction can already
be observed. A maximum degree of reduction was reached 20–30 min after
the addition of PMA.

3 is an average over 15 cells, and in Fig. 3, E and F, the
result is an average over five measurements on different
cells. Fig. 3 D contains bands at 1473, 1597, 1606, and 1615
cm
1
that are specific for a reduction of MPO. Furthermore,
the bands at 1528 and 1580 cm
1
indicate a reduction of
cytochrome \( b_{558} \). Comparison with the intensities in Fig. 3
A shows that for healthy donors \( \sim 45 \pm 10\% \) of the MPO
and 30 \( \pm 10\% \) of the cytochrome \( b_{558} \) were in the reduced
state after activation with PMA (a 100% change being
obtained after the addition of dithionite). The same reactions
upon PMA activation were observed in four batches of
normal neutrophils isolated on different days from two
different donors. The difference spectrum of MPO-deficient
neutrophils (Fig. 3 E) shows a reduction of \( \sim 20\% \) of the
cytochrome \( b_{558} \). A reduction of cytochrome \( b_{558} \) was seen
in 40 measurements on different PMA-activated MPO-de-
cient neutrophils. In the difference spectrum of the CGD
neutrophils (Fig. 3 F) no reaction can be observed. We have
measured 60 different CGD neutrophils after PMA activa-
tion, and in none of them was a reaction visible. The spectra
were measured between 10 and 40 min after the addition
of PMA. Over 100 control neutrophils of the normal, MPO-
deficient, and CGD donors that were not activated with
PMA were measured until 1 h after preparation. No reac-
tions were observed in these control samples.
Activation of neutrophilic granulocytes by PMA triggers
NADPH oxidase activity and, therefore, the formation of
oxygen metabolites. This event is monitored by our tech-
nique from the changes in redox state of the heme group of
cytochrome \( b_{558} \). A reduction of MPO can be observed if
both MPO and cytochrome \( b_{558} \) are present in the cell. In
the absence of cytochrome \( b_{558} \) no reduction of MPO can be
observed.
The results indicate that the formation of oxygen metab-
olites is necessary to obtain a reduction of MPO. This
supports the observation that absorption measurements on
PMA-activated normal neutrophils under anaerobic condi-
tions did not show a reduction of MPO (Cross et al., 1982;
Iizuka et al., 1985). The results can be understood in terms
of a model reported by Winterbourn et al. (1985). Activa-
tion of the NADPH-oxidase complex leads to oxygen con-
sumption and the formation of \( O_2^- \) or \( H_2O_2 \). The oxygen
metabolite reacts with MPO to form compound III
(\( MPO^2+O_2 \)), which then converts to \( MPO^{2+} \) (reduced
MPO) upon deoxygenation.
In the confocal Raman measurements a much higher
degree of MPO reduction was observed than in the absorp-
tion measurements. This, we believe, results from the much
higher \( (\sim 5 \times 10^3 \times) \) oxygen-to-cell ratio in the single cell
Raman measurements than in the absorption measurements
on a dense suspension of cells.
A reduction of cytochrome \( b_{558} \) was observed in the
confocal Raman spectra measured inside PMA-activated
neutrophils. Because of the high spatial resolution of the
method, this observation indicates intracellular NADPH
oxidase activity. However, Ambruso and co-authors (1990)
have estimated that after PMA activation 63% of the cyto-
chrome \( b_{558} \) is located in the plasma membrane and 31% in
the specific granules. Because of this much larger cyto-
chrome \( b_{558} \) concentration in the plasma membrane it can be
argued that a small fraction of out-of-focus light may con-
tribute to the signal in the confocal Raman microspectra
measured inside the neutrophils.
However, the fact that a reduction of MPO was also
observed is an additional indication of intracellular NADPH
oxidase activity. The MPO signal certainly originates from
MPO located in the azurophilic granules. The Raman mea-
surements were performed with only \( \sim 2 \times 10^4 \) neutrophils
on a quartz slide in 2 ml buffer solution. If degranulation of
the neutrophils occurs, MPO will be released in the buffer
solution. However, the maximum extracellular MPO con-
centration that can be reached if 100% of the MPO is
released is over \( 10^5 \) times less than the intracellular MPO
concentration, because the buffer volume is much larger
than the cell volume. The Raman signal of such a low
extracellular MPO concentration could not have been ob-
served under our measurement conditions, and we conclude
that the Raman signal can only come from intracellular
MPO.
Another important observation is that no reduction of
MPO was observed in the cytochrome \( b_{558} \)-deficient neu-
rophils. The only difference between these neutrophils and
normal neutrophils was the absence of cytochrome \( b_{558} \),
which made them incapable of forming oxygen metabolites.

Biophysical Journal 78(5) 2606–2613
That no reduced MPO was formed in these cells is strong evidence for the necessity of oxygen metabolites for the formation of reduced MPO. The oxygen metabolites can only come in contact with the MPO located in the azurophilic granules if the NADPH oxidase activity takes place in the same organelle or in very close contact with the azurophilic granules, because of the enormous capacity of the cytosol to consume hydrogen peroxide.

The observation of intracellular NADPH oxidase activity in PMA-activated neutrophils is in agreement with recent results obtained with other techniques (Lundqvist et al., 1996; Kobayashi et al., 1998; Jankowski and Grinstein, 1999; Vaisierie et al., 1999). Most techniques used in these papers demand chemical treatment of the neutrophils like fixation, labeling, or fractioning, whereas our results were obtained with living neutrophils under physiological conditions.

We have also measured the reactions taking place in PMA-activated eosinophilic granulocytes. It is known that in eosinophilic granulocytes reactions occur that are similar to those in neutrophilic granulocytes with eosinophilic peroxidase (EPO) instead of MPO as the redox center (Wever and Plat, 1981). In Fig. 4 resonance Raman spectra of normal (Fig. 4 A) and CGD (Fig. 4 D) eosinophils with reduced redox centers are compared with spectra of normal (Fig. 4 C) and CGD (Fig. 4 D) eosinophils with oxidized redox centers. It is clear that the spectra of the normal and CGD eosinophils are almost identical. The spectra of the native eosinophils correspond very well with published spectra of EPO. Apparently the Raman signal from the cytoplasmic region of eosinophils mainly originates from EPO.

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FIGURE 3 Difference spectra of neutrophils with reduced minus neutrophils with oxidized redox centers of (A) a normal, (B) a MPO-deficient, and (C) a cytochrome b558-deficient donor are compared with difference spectra of PMA-activated minus control neutrophils of a (D) normal, (E) a MPO-deficient, and (F) a cytochrome b558-deficient donor. Difference spectra were calculated from an average of 15 (A–D) or five (F and G) measurements on different cells. Positions of the most important bands in A–C are summarized in Table 1.

<table>
<thead>
<tr>
<th>Band/cm⁻¹</th>
<th>Normal NG</th>
<th>MPO-def. NG</th>
<th>CGD NG</th>
</tr>
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<tbody>
<tr>
<td>1360</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1375</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
</tr>
<tr>
<td>1473</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>1528</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>1553</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>1564</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1580</td>
<td>−−</td>
<td>−−</td>
<td>0</td>
</tr>
<tr>
<td>1597</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>1606</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>1615</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
</tbody>
</table>

++ and −− indicate a relatively strong positive or negative band, + and − a positive or negative band, and 0 indicates that no band can be recognized. Bands specific for cytochrome b558 are a positive band at 1528 and a negative band at 1580 cm⁻¹. MPO-specific bands are positive bands at 1473 and 1606 cm⁻¹ and negative bands at 1553, 1597, and 1615 cm⁻¹.
EPO. The difference spectrum of PMA-activated eosinophils (Fig. 4F) corresponds very well with the spectrum of reduced CGD eosinophils (Fig. 4G). This indicates that a reduction of EPO has occurred upon PMA activation. About 70% of the EPO was in the reduced state after activation. Minor differences can be recognized between Fig. 4E and G: in Fig. 4E an extra band at 1525 cm\(^{-1}\) is visible on top of the broad band around 1430 cm\(^{-1}\) that is also present in Fig. 4G. Furthermore, at the right side from the 1560 cm\(^{-1}\) band in Fig. 4E a shoulder around 1580 cm\(^{-1}\) can be recognized. The intensity of the 1360/1375 cm\(^{-1}\) band in Fig. 4G is only 67% of the intensity in Fig. 4E. These differences correspond to the difference spectrum of reduced minus oxidized cytochrome \(b_{558}\). In the difference spectra of PMA-activated eosinophils (after scaling on the 1473 cm\(^{-1}\) band of reduced EPO) the intensity of the 1360/1375 cm\(^{-1}\) bands is comparable to the intensity in Fig. 4E, which indicates that a reduction of cytochrome \(b_{558}\) has also occurred upon PMA activation.

The spectral changes in eosinophils occurring after PMA activation are similar to those observed after activation of eosinophilic granulocytes with opsonized polystyrene spheres (Puppels et al., 1995). The percentage of EPO that has been reduced after PMA activation of eosinophils was almost twice that of reduced MPO after activation of neutrophils, which correlates well with the larger NADPH oxidase activity in PMA-activated eosinophils.

Our results with PMA-activated eosinophilic granulocytes confirm our interpretation of the Raman spectra of neutrophils. In the eosinophils a reduction of EPO was observed upon PMA activation, whereas the neutrophils showed a reduction of MPO. This is in agreement with the expectation, because similar reactions should take place upon PMA activation in both granulocytes with EPO as the redox center in the eosinophils and MPO in the neutrophils.

**CONCLUSIONS**

Resonant Raman spectra from a small volume in PMA-activated neutrophilic granulocytes show a reduction of both MPO and cytochrome \(b_{558}\), whereas PMA-activated cytochrome \(b_{558}\)-deficient neutrophils do not show any reaction at all. These results can only be explained by an intracellular NADPH oxidase activity. The exact location of the NADPH oxidase activity cannot be determined with our method. However, the oxygen metabolites should be formed close to the azurophilic granulocytes, because of the large capacity of the cytosol to consume hydrogen peroxide.

Resonant Raman measurements of PMA-activated eosinophilic granulocytes show an intracellular reduction of EPO. This result corroborates the results obtained with the neutrophilic granulocytes.
REFERENCES


Sijtsema et al.

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