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

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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 azurophilic 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):

$$\begin{aligned} \mathrm{MPO} + \mathrm{H_2O_2} &\rightarrow \text{Compound I} + \mathrm{H_2O} \\ \text{Compound I} + \mathrm{H^+} + \mathrm{Cl^-} &\rightarrow \mathrm{MPO} + \mathrm{HOCl} \end{aligned}$$

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

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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 O_2^- and H_2O_2 hardly affected the PMA-induced chemiluminescence response in human neutrophils, indicating an intracellular localization of the activity (Vaissiere 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, compound III, reduced MPO, and reduced cytochrome b_{558} under aerobic conditions (Winterbourn et al., 1985). However, from these results it cannot be concluded whether these products were formed intracellularly or extracellularly.

Confocal Raman microspectroscopy is a method that combines molecular specificity with diffraction-limited resolution (Puppels et al., 1991a). A biological sample can be investigated under physiological conditions. Raman spectra contain information about the chemical composition of the measurement volume. The small probe volume localizes the position of reactions, e.g., in the cytoplasm or in the nucleus or at the plasma membrane. In this way it is possible to study the cellular response to a disturbance.

Raman microspectra for tissue (Manoharan et al., 1996), chromosomes (Puppels et al., 1992), or specific parts of living cells (Puppels et al., 1990, 1993) have been published. It was shown that it is feasible to measure spectra of MPO in living neutrophils (Puppels et al., 1991b) and of EPO in living eosinophilic granulocytes (Salmaso et al., 1994). Resonant Raman spectra of isolated MPO (Babcock et al., 1985; Floris et al., 1995; Sibbett and Hurst, 1984; Stump et al., 1987), MPO compound II (Oertling et al., 1988), and EPO (Sibbett et al., 1985), as well as spectra of isolated oxidized and reduced cytochrome b_{558} (Hurst et al., 1991), show that the different compounds can be distinguished based on their Raman spectra. In a previous publication (Sijtsema et al., 1998a) we have shown that resonant Raman spectra (413-nm excitation) measured in the cytoplasm of living neutrophils contain prominent Raman bands of oxidized MPO and cytochrome b_{558}. The contributions of other redox states of MPO and cytochrome b_{558} could be observed after the addition of (reducing) sodium dithionite. 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 neutrophilic 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 (2×10^6) 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 neutrophilic 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 5×10^7 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 (A(sample) $-$ A(suspension of native neutrophils)).

In both the Raman and the absorption measurements the neutrophils were activated by the addition of 0.1 μg/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 μm) 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 24×24 μm^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 μm in the lateral direction and 1.2 μm in the axial direction for a small sphere (diameter 0.282 μm) and 3.6 μm in the axial direction for a thin layer (thickness 0.28 μm) (Sijtsema et al., 1998b). The axial resolution for cellular samples will be between 1.2 and 3.6 μm. The spectral resolution was $\sim$4 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 neutrophilic 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 (λ_{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.

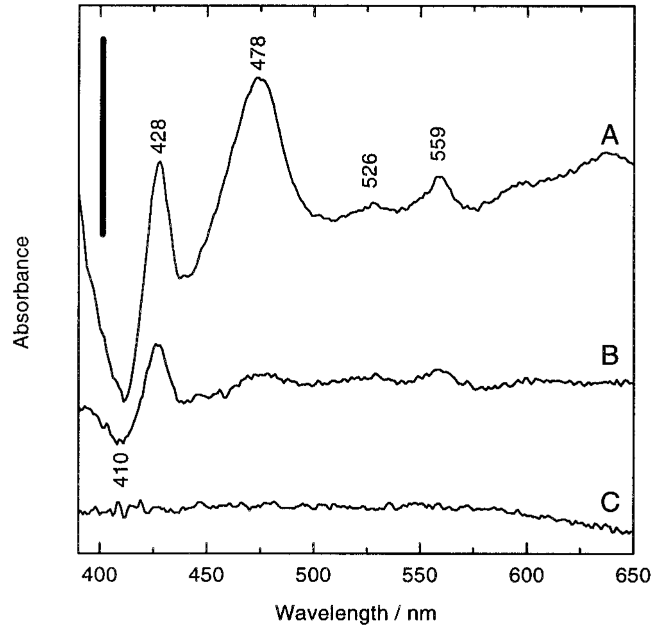

FIGURE 1 Absorption difference spectrum of (*A*) sodium dithionite-treated and (*B* and *C*) PMA-activated activated neutrophils with native neutrophils of a healthy donor. *B* and *C* were measured 11 and 3 min, respectively, after the addition of PMA. The negative bands at 410 and 577 nm in combination with the positive bands at 428 and 560 nm in *A* and *B* indicate a reduction of cytochrome b_{558}. The band at 470 nm is specific for a reduction of MPO. The bar indicated in the figure corresponds to an absorbance of 0.05.

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 granulomatous disease (CGD) patient (Fig. 3 *F*) are given. The result in Fig.

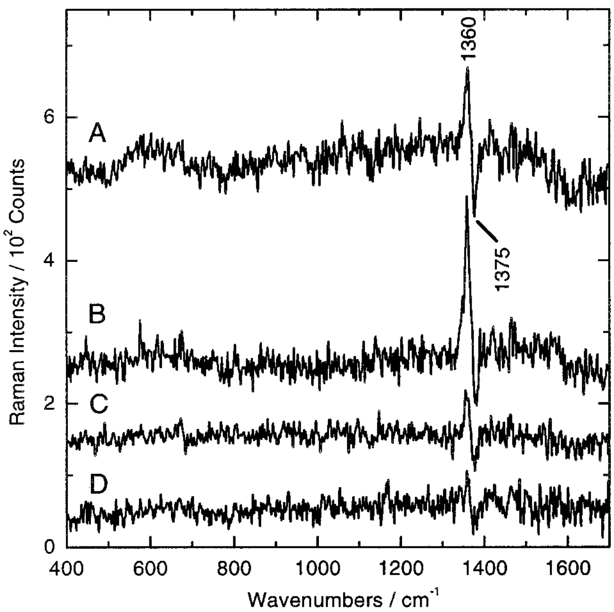

FIGURE 2 Raman difference spectra of single PMA-activated neutrophils measured at (*A*) 32, (*B*) 28, (*C*) 16, and (*D*) 14 min after the addition of PMA. Fourteen minutes after the addition of PMA a reaction can already be observed. A maximum degree of reduction was reached 20–30 min after the addition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

3 *D* 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 ~45 ± 10% of the MPO and 30 ± 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 ~20% of the cytochrome b_{558}. A reduction of cytochrome b_{558} was seen in 40 measurements on different PMA-activated MPO-deficient 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 activation, 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 reactions 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 technique 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 metabolites is necessary to obtain a reduction of MPO. This supports the observation that absorption measurements on PMA-activated normal neutrophils under anaerobic conditions 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). Activation of the NADPH-oxidase complex leads to oxygen consumption 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 absorption measurements. This, we believe, results from the much higher (~$5 \times 10^3 \times$) 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 cytochrome b_{558} is located in the plasma membrane and 31% in the specific granules. Because of this much larger cytochrome b_{558} concentration in the plasma membrane it can be argued that a small fraction of out-of-focus light may contribute 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 measurements were performed with only ~2×10^4 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 concentration 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 observed 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 neutrophils. The only difference between these neutrophils and normal neutrophils was the absence of cytochrome b_{558}, which made them incapable of forming oxygen metabolites.

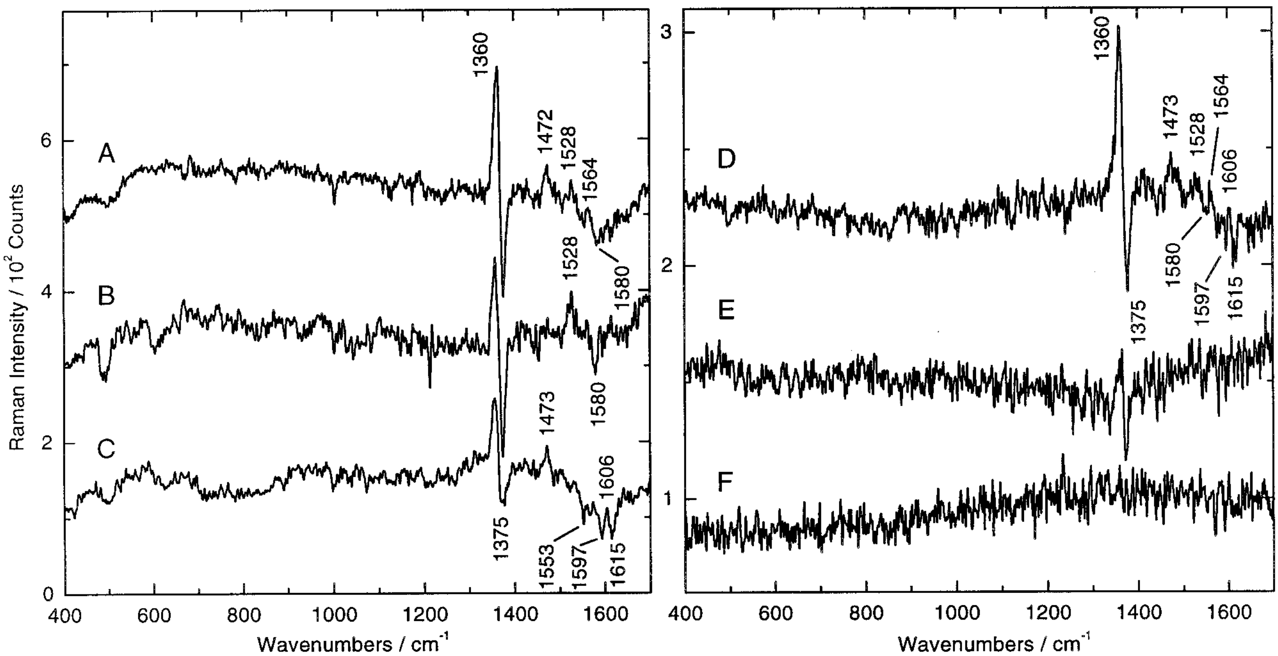

FIGURE 3 Difference spectra of neutrophils with reduced minus neutrophils with oxidized redox centers of (*A*) a normal, (*B*) a MPO-deficient, and (*C*) a cytochrome b_{558}-deficient donor are compared with difference spectra of PMA-activated minus control neutrophils of a (*D*) normal, (*E*) a MPO-deficient, and (*F*) a cytochrome b_{558}-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.

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.

TABLE 1 A comparison of the Raman bands in the difference spectra of reduced minus oxidized normal neutrophilic granulocytes (NG), MPO-deficient NG, and CGD NG (Fig. 3, *A–C*)

Band/ cm^{-1}	Normal NG	MPO-def. NG	CGD NG
1360	++	++	++
1375	−−	−−	−−
1473	+	0	+
1528	+	+	0
1553	−	0	−
1564	+	+	+
1580	−	−	0
1597	−	0	−
1606	+	0	+
1615	−	0	−

++ 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 b_{558} 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}.

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; Vaissiere 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 *B*) 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.

Fig. 4 presents the difference spectra of dithionite-reduced (Fig. 4 E) and PMA-activated (Fig. 4 *F*) normal eosinophils together with the difference spectrum of CGD eosinophils (Fig. 4 *G*) with reduced redox centers. The difference spectra are scaled on the 1473 cm^{-1} band of

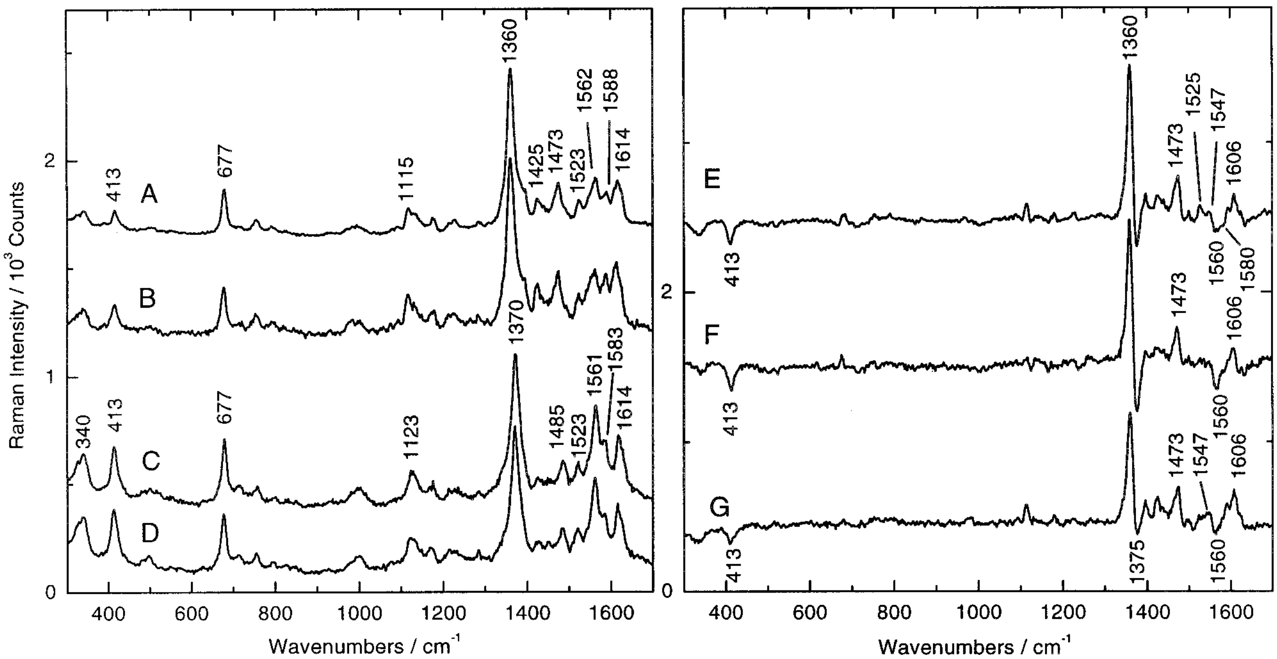

FIGURE 4 Resonant Raman spectra of the cytoplasmic region of (*A*) normal and (*B*) CGD eosinophils after reduction of the redox centers and of (*C*) normal and (*D*) CGD eosinophils with oxidized redox centers. Spectra were averaged over five measurements on different cells. Signals in *A* and *B* were divided by 2. Furthermore, difference spectra of (*E*) dithionite-reduced minus native eosinophils as well as (*F*) PMA-activated minus native eosinophils of a normal donor are compared with the difference spectrum of (*G*) dithionite-reduced minus native eosinophils of a CGD donor. The difference spectra were scaled on the 1473 cm^{-1} band of reduced EPO.

EPO. The difference spectrum of PMA-activated eosinophils (Fig. 4 *F*) corresponds very well with the spectrum of reduced CGD eosinophils (Fig. 4 *G*). 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. 4, *E* and *G*: in Fig. 4 *E* 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. 4 *G*. Furthermore, at the right side from the 1560 cm^{-1} band in Fig. 4 *E* a shoulder around 1580 cm^{-1} can be recognized. The intensity of the 1360/1375 cm^{-1} band in Fig. 4 *G* is only 67% of the intensity in Fig. 4 *E*. 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 EPO) the intensity of the 1360/1375 cm^{-1} bands is comparable to the intensity in Fig. 4 *E*, 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.

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REFERENCES

Ambruso, D. R., B. G. J. M. Bolscher, P. M. Stokman, A. J. Verhoeven, and D. Roos. 1990. Assembly and activation of the NADPH-O_2^- oxidoreductase in human neutrophils after stimulation with phorbol-myristate acetate. *J. Biol. Chem.* 265:924–930.

Babcock, G. T., R. T. Ingle, W. A. Oertling, J. C. Davis, B. A. Averill, C. L. Hulse, D. J. Stufkens, B. G. J. M. Boscher, and R. Wever. 1985. Raman characterization of human leukocyte myeloperoxidase and bovine spleen green haemoprotein. Insight into chromophore structure and evidence that the chromophores of myeloperoxidase are equivalent. *Biochim. Biophys. Acta.* 828:58–66.

Babior, B. M. 1984. Oxidants from phagocytes: agents of defence and destruction. *Blood.* 64:959–966.

Baehner, R. L., and D. G. Nathan. 1967. Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science.* 155:835.

Bellavite, P. 1988. The superoxide-forming enzymatic system of the phagocytes. *Free Radic. Biol. Med.* 4:225–261.

Borregaard, N. 1988. The human neutrophil. Function and dysfunction. *Eur. J. Haematol.* 41:401–413.

Clark, R. A., B. D. Volpp, K. G. Leidal, and W. M. Nauseef. 1990. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J. Clin. Invest.* 85:714–721.

Cross, A. R., and J. T. Curnutte. 1995. The cytosolic activating factors $p47^{phox}$ and $p67^{phox}$ have distinct roles in the regulation of electron flow in NADPH oxidase. *J. Biol. Chem.* 270:6543–6548.

Cross, A. R., F. K. Higson, and O. T. G. Jones. 1982. The enzymic reduction and kinetics of oxidation of cytochrome b_{-245} of neutrophils. *Biochem. J.* 204:479–485.

Curnutte, J. T., D. M. Whitten, and B. M. Babior. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N. Engl. J. Med.* 290:593–597.

Dusi, S., and F. Rossi. 1993. Activation of NADPH-oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox. *Biochem. J.* 296:367–371.

Floris, R., N. Moguilevsky, G. Puppels, V. Deleersnyder, A. Jacquet, L. Garcia-Quintana, R. Renirie, A. Bollen, and R. Wever. 1995. Heme-protein interaction in myeloperoxidase: modification of spectroscopic properties and catalytic activity by single residue mutation. *J. Am. Chem. Soc.* 117:3907–3912.

Henderson, W. R. 1991. Eosinophil peroxidase: occurrence and biological function. *In* Peroxidases in Chemistry and Biology. J. Everse, K. E. Everse, and M. B. Grisham, editors. CRC Press, Boca Raton, FL. 105–122.

Hurst, J. K., T. M. Loehr, J. T. Curnutte, and H. Rosen. 1991. Resonance Raman and electron paramagnetic resonance structural investigations of neutrophil cytochrome b_{558}. *J. Biol. Chem* 266:1627–1634.

Iizuka, T., S. Kanegasaki, R. Makino, T. Tanaka, and Y. Ishimura. 1985. Studies on neutrophil *b*-type cytochrome in situ by low temperature absorption spectroscopy. *J. Biol. Chem.* 260:12049–12053.

Jankowski, A., and S. Grinstein. 1999. A noninvasive fluorimetric procedure for measurement of membrane potential. Quantification of the NADPH oxidase-induced depolarization in activated neutrophils. *J. Biol. Chem.* 274:26098–26104.

Klebanoff, S. J. 1991. Myeloperoxidase: occurrence and biological function. *In* Peroxidases in Chemistry and Biology. J. Everse, K. E. Everse, and M. B. Grisham, editors. CRC Press, Boca Raton, FL. 1–36.

Klebanoff, S. J., J. M. Agost, A. Jorg, and A. M. Waltersdorph. 1989. Comparative toxicity of the horse eosinophil peroxidase-H_2O_2-halide system and granule basic proteins. *J. Immunol.* 143:239–244.

Knaus, U. G., P. G. Heyworth, T. Evans, J. T. Curnutte, and G. M. Bokoch. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac-2. *Science.* 254:1512–1515.

Kobayashi, T., J. M. Robinson, and H. Seguchi. 1998. Identification of intracellular sites of superoxide production in stimulated neutrophils. *J. Cell Sci.* 111:81–91.

Kooter, I. M., N. Moguilevsky, A. Bollen, N. M. Sijtsema, C. Otto, and R. Wever 1997. Site-directed mutagenesis of Met243, a residue of myeloperoxidase involved in the binding of the prosthetic group. *J. Biol. Inorg. Chem.* 2:191–197.

Lehrer, R. I., and M. J. Cline. 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis—role of myeloperoxidase in resistance to *Candida* infection. *J. Clin. Invest.* 48:1478–1488.

Lundqvist, H., P. Follin, L. Khalfan, and C. Dahlgren. 1996. Phorbol myristate acetate-induced NADPH-oxidase activity in human neutrophils: only half the story has been told. *J. Leukoc. Biol.* 59: 270–279.

Majumdar, S., M. W. Rossi, T. Fujiki, W. A. Philips, S. Disa, C. F. Queen, R. B. Johnston Jr, O. M. Rosen, B. E. Corkey, and H. M. Korchak. 1991. Protein kinase C isotypes and signaling in neutrophils. Differential substrate specificities of a translocatable, calcium- and phospholipid-dependent β-protein kinase C and a novel calcium-independent, phospholipid-dependent protein kinase which is inhibited by long chain fatty acyl coenzyme A. *J. Biol. Chem.* 266:9285–9295.

Manoharan, R., Y. Wang, and M. S. Feld. 1996. Histochemical analysis of biological tissues using Raman spectroscopy. *Spectrochim. Acta Part A.* 52:215–249.

Nauseef, W. M. 1990. Myeloperoxidase deficiency. *Hematol. Pathol.* 4:165–178.

Oertling, W. A., H. Hoogland, G. T. Babcock, and R. Wever. 1988. Identification and properties of an oxoferryl structure in myeloperoxidase compound II. *Biochemistry.* 27:5395–5400.

Puppels, G. J., T. C. Bakker Schut, N. M. Sijtsema, M. Grond, F. Maraboeuf, C. G. de Grauw, C. G. Figdor, and J. Greve. 1995. Development and application of Raman microspectroscopic and Raman imaging techniques for cell biological studies. *J. Mol. Struct.* 347:477–484.

Puppels, G. J., W. Colier, J. H. F. Olminkhof, C. Otto, F. F. M. de Mul, and J. Greve. 1991a. Description of a highly sensitive confocal Raman microspectrometer. *J. Raman Spectrosc.* 22:217–225.

Puppels, G. J., F. F. M. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin, and T. M. Jovin. 1990. Studying single living cells and chromosomes by confocal Raman microspectroscopy. *Nature.* 347: 301–303.

Puppels, G. J., H. S. P. Garritsen, J. A. Kummer, and J. Greve. 1993. Carotenoids located in human lymphocyte subpopulations and natural killer cells by Raman microspectroscopy. *Cytometry.* 14:251–256.

Puppels, G. J., H. S. P. Garritsen, G. M. J. Segers-Nolten, F. F. M. de Mul, and J. Greve. 1991b. Raman microspectroscopic approach to the study of human granulocytes. *Biophys. J.* 60:1046–1056.

Puppels, G. J., C. A. J. Putman, B. G. de Grooth, and J. Greve. 1992. Raman microspectroscopy of chromosomal banding patterns. *SPIE.* 1922:145–155.

Rossi, F. 1986. The O_2^--forming NADPH-oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67phox. *Biochim. Biophys. Acta.* 853:65–98.

Rotrosen, D., C. L. Yeung, T. L. Leto, H. L. Malech, and C. H. Kwong. 1992. Cytochrome b_{558}—the flavin binding component of the phagocyte NADPH-oxidase. *Science.* 256:1459–1462.

Salmaso, B. L. N., G. J. Puppels, P. J. Caspers, R. Floris, R. Wever, and J. Greve. 1994. Resonance Raman microspectroscopic characterization of eosinophil peroxidase in human eosinophilic granulocytes. *Biophys. J.* 67:436–446.

Segal, A. W., I. West, F. Wientjes, J. H. A. Nugent, A. J. Chavan, B. Haley, R. C. Garcia, H. Rosen, and G. Scrace. 1992. Cytochrome-b_{-245} is a flavocytochrome containing Fad and the NADPH-binding site of the microbicidal oxidase of phagocytes. *Biochem. J.* 284:781–788.

Sibbett, S. S., and J. K. Hurst. 1984. Structural analysis of myeloperoxidase by resonance Raman spectroscopy. *Biochemistry.* 23:3007–3013.

Sibbett, S. S., S. J. Klebanoff, and J. K. Hurst. 1985. Characterization of the heme prosthetic group in eosinophil peroxidase. *FEBS Lett.* 189: 271–275.

Sijtsema, N. M., C. Otto, G. M. J. Segers-Nolten, A. J. Verhoeven, and J. Greve. 1998b. Resonance Raman microspectroscopy of myeloperoxidase and cytochrome b_{558} in human neutrophilic granulocytes. *Biophys. J.* 74:3250–3255.

Sijtsema, N. M., S. D. Wouters, C. J. de Grauw, C. Otto, and J. Greve. 1998a. A confocal direct imaging Raman microscope: design and applications in biology. *Appl. Spectrosc.* 52:348–355.

Smith, R. M., and J. T. Curnutte. 1991. Molecular basis of chronic granulomatous disease. *Blood.* 77:673–686.

Stump, R. F., G. G. Deanin, J. M. Oliver, and J. A. Shelnutt. 1987. Heme-linked ionizations of myeloperoxidase detected by Raman difference spectroscopy. A comparison with plant and yeast peroxidases. *Biophys. J.* 51:605–610.

Sumimoto, H., N. Sakamoto, M. Nozaki, Y. Sakaki, K. Takeshige, and S. Minakami. 1992. Cytochrome b_{558}, a component of the phagocyte NADPH-oxidase, is a flavoprotein. *Biochem. Biophys. Res. Commun.* 186:1368–1375.

Test, S. T., and S. J. Weiss. 1984. Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *J. Biol. Chem.* 259:399–405.

Vaissiere, C., V. Le Cabec, E. Faure, and I. Maridonneau-Parini. 1995. Intracellular generation of O_2^- in activated neutrophils: assembly of the NADPH-oxidase in the membrane of specific granules. *Eur. J. Clin. Invest.* 25:A21.

Vaissiere, C., V. Le Cabec, and I. Maridonneau-Parini. 1999. NADPH oxidase is functionally assembled in specific granules during activation of human neutrophils. *J. Leukoc. Biol.* 65:629–634.

Wever, R., and H. Plat. 1981. Spectral properties of myeloperoxidase and its ligand complexes. *Biochim. Biophys. Acta.* 661:235–239.

Winterbourn, C. C., R. C. Garcia, and A. W. Segal. 1985. Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated human neutrophils and its reactivity with hydrogen peroxide and chloride. *Biochem. J.* 228:583–592.

Yazdanbakhsh, M., C. M. Eckmann, L. Koenderman, A. J. Verhoeven, and D. Roos. 1987. Eosinophils do respond to fMLP. *Blood.* 70:379–383.