Identification of a superoxide-generating NADPH oxidase system in human fibroblasts

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Human fibroblasts have the capacity to release superoxide radicals upon stimulation of an electron transport system similar to the NADPH oxidase of leukocytes. Two components of the NADPH oxidase system, (1) a flavoprotein of 45 kDa which binds diphenylene iodonium (a compound described as a specific inhibitor of the leukocyte NADPH oxidase), and (2) a low-potential cytochrome *b*, are present in fibroblast membranes. Fibroblasts exhibit these compounds at lower concentrations than do polymorphonuclear leukocytes or B-lymphocytes. The superoxide-generating system is rather uniformly associated with the outer cell membrane, as shown by light and electron microscopy. Superoxide release upon stimulation with various agents was prevented by the addition of micromolar concentrations of diphenylene iodonium, making an NADPH oxidase a likely source.

INTRODUCTION

The release of reactive oxygen species by leucocytes [neutrophil and eosinophil polymorphonuclear cells (PMNs), monocytes and B-lymphocytes] upon stimulation is well documented as an important defence mechanism against infectious agents, but tissue cells were originally thought not to release reactive oxygen species. However, it is now recognized that numerous cell types also are able to release reactive oxygen species upon stimulation. These include endothelial cells [1–3], fibroblasts [4–6], mesangial cells [7–9], epithelial cells [10], cells of the corpora lutea [11], smooth muscle cells [12] and platelets [13,14].

Leucocytes generate superoxide anions upon contact with appropriate stimuli by means of a membrane-bound NADPH oxidase system possessing a flavoprotein of 45 kDa, with non-covalent bound FAD as prosthetic group and a cytochrome *b* [15–21]. The cytochrome *b* exhibits an unusually low redox potential of $E_{m,7.0}$ (midpoint potential) = −245 mV [21], capable of reducing oxygen to superoxide.

Superoxide generation is specifically inhibited by diphenyleneiodonium (DPI), which binds at low concentrations to the flavoprotein compound of the NADPH oxidase [22–24].

The superoxide-generating system in non-leucocytes has not been identified, but several indirect indications suggest the involvement of an NADPH oxidase system, similar to that in leucocytes. Superoxide generation is not stimulated by xanthine or inhibited by allopurinol, excluding the participation of the xanthine oxidase system. On the other hand, superoxide release increases upon addition of NADH or NADPH, whereas rotenone and azide have no inhibitory effects, excluding the mitochondrial electron transport chain as a possible source [4,5].

The present paper gives evidence that superoxide release by fibroblasts is caused by an NADPH oxidase system possessing strong similarity to the NADPH oxidase of phagocytes.

MATERIALS AND METHODS

RPMI medium (R 10 SP) and trypsin (0.125%, w/v)/EDTA (0.01%, w/v) were obtained from Gibco BRL (Eggestein, Germany); fetal calf serum (FCS; lot no. 14K03) was from Biochrom (Berlin, Germany); sterile plastic material for cell cultures was from Nunc (Wiesbaden, Germany) and plastic cover slides (22 mm × 60 mm) and 4-well multiplates were from Lux (Newbury Park, CA, U.S.A.). Cytochrome *c* grade III, peroxidase type III, Zymosan A (lot no. 86F-0666) phorbol 12-myristate 13-acetate (PMA), di-isopropyl fluorophosphate, phenazine methosulphate, phenazine ethosulphate, duroquinone, Mops and molecular mass standards (SDS-7) were from Sigma (Deisenhoven, Germany). The Ca^{2+} ionophore A23187 was from Calbiochem (Frankfurt, Germany); pyocyanine was from K & K Laboratories (Liverpool, U.K.); 2-hydroxy-1,4-naphthoquinone, anthraquinone-2,6-disulphonate, anthraquinone and 3-amino-1,2,4-triazole were from Aldrich (Steinheim, Germany); 3,6-diaminodurene was from Fluka (Derby, U.K.) and NitroBlue Tetrazolium (NBT) and scopoletin were from Serva (Heidelberg, Germany). Interleukin-1 was obtained from Dainippon (Tokyo, Japan); tumour necrosis factor was kindly supplied by Bissendorf Peptid (Wedemark, Germany) and superoxide dismutase by Grünenthal (Aachen, Germany). DPI and [^{125}I]DPI were synthesized as described [25,26]. Na^{125}I was obtained from Amersham International (Amersham, Bucks., U.K.). The concentrations of DPI solutions were determined photometrically using a molar absorption coefficient at 264 nm (ϵ_{264}) of 15.1 $mM^{-1} \cdot cm^{-1}$. All other chemicals were from Merck (Darmstadt, Germany).

Cell cultures

Primary cultures of human fibroblasts were established by the method of explant cultures and propagated in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated FCS in air/CO_2 (19:1) at 37 °C as described [4]. To avoid even low contamination by phagocytes, only cells of the 10th to 20th passage were used for the experiments. For determination of $O_2^{\cdot-}$ and H_2O_2 production, the fibroblasts were cultured as monolayers on all four sides of glass cuvettes or on plastic slides in 4-multiwell chambers [4] for electron microscopy.

Abbreviations used: DPI, diphenyleneiodonium; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; NBT, NitroBlue Tetrazolium; PMN, polymorphonuclear cell; $E_{m,7.0}$, midpoint potential.

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Serum treatment of zymosan

Zymosan was washed with phosphate buffer (50 mmol/l, pH 7.4) containing NaCl (0.15 mol/l), heated at 99 °C for 90 min and washed again three times. A 1 ml portion of zymosan solution (10 mg/ml) was added to 3 ml of fresh human serum and incubated for 30 min at 37 °C with gentle shaking. The solution was then washed twice with the phosphate buffer, resuspended to a concentration of 10 mg/ml and stored frozen until used.

Preparation of fibroblast membranes

Fibroblasts were detached with trypsin/EDTA (0.125%/0.01%, w/v) and 5 ml of RPMI containing 10% (v/v) FCS was added per flask (80 cm^2) to stop the reaction. The cells were sedimented by centrifugation (1000 *g*, 10 min), washed twice with test buffer (see below) and sonicated with 4 × 15 s bursts with 15 s intervals at 0 °C in phosphate-buffered saline containing di-isopropyl fluorophosphate (2 mmol/l) using a Branson-185 sonifier at 50% power. Fibroblast membranes were precipitated by ultracentrifugation (105000 *g* for 60 min).

Determination of the cytochrome b_{-245} content

Reduced-minus-oxidized difference spectra of the fibroblast membranes and spectra at a known redox potential were recorded with a rapid-scanning split-beam spectrophotometer as described [21]. Reduction was performed anaerobically in KCl (100 mmol/l)/Mops (50 mmol/l) buffer at pH 7.0 with the following mediators: phenazine methosulphate (12.5 μmol/l), phenazine ethosulphate (12.5 μmol/l), pyocyanine (6 μmol/l), 2-hydroxy-1,4-naphthoquinone (12.5 μmol/l), anthraquinone-2,6-disulphonate (12.5 μmol/l), anthraquinone (12.5 μmol/l), 3,6-diaminodurene (12.5 μmol/l) and duroquinone (12.5 μmol/l). The reduction potential was decreased by the addition of small amounts of sodium dithionite solution and increased by addition of potassium ferricyanide solution, and was determined anaerobically in a stirred cuvette fitted with platinum and calomel electrodes as described [27]. The titrations for determination of mid-point potential were performed in an anaerobic box fluxed with N_2 by adjusting the potential as described above. The solution (2 ml) was anaerobically transferred to a cuvette closed with a Teflon stopper, and spectra were recorded with a Beckman ACTA III spectrophotometer (Beckman, Irvine, CA, U.S.A.). The reference cuvette contained an identical membrane suspension without added mediators or reductants. Complete oxidation of cytochromes was ensured by adding ferricyanide to the reference. The absolute amount of cytochrome *b* and low-potential cytochrome *b* at different redox potentials was calculated by measuring the absorption at 559 nm using $\epsilon_{559-540} = 21.6\ mM^{-1} \cdot cm^{-1}$.

Radioactive labelling of the flavoprotein

The fibroblast membranes were incubated for 10 min at 20 °C with [^{125}I]DPI, solubilized in SDS buffer (pH 6.8) containing Tris/HCl (50 mmol/l), 1% (w/v) SDS, 1.25% (v/v) mercaptoethanol and 8% (v/v) glycerol, and proteins were separated by electrophoresis on 12% (w/v)-polyacrylamide gels. For autoradiography the gel was fixed in 20% (v/v) methanol and afterwards in 20% (v/v) methanol/5% (v/v) glycerol, each for 2 h. The gel was then dried overnight and autoradiographed against a Fuji-Rx film for 3 days and scanned. Molecular mass was determined by using standards (Sigma, SDS-7) with the following protein markers: BSA, 66 kDa; egg albumin, 45 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; lactalbumin, 14.2 kDa.

Determination of superoxide formation

Fibroblasts were cultured to confluency (5×10^4 cells/cm^2) in cuvettes as described as monolayers on all four sides of glass cuvettes. The activity measurements were performed in 2 ml of potassium phosphate buffer (50 mmol/l, pH 7.2) supplemented with NaCl (150 mmol/l), $MgCl_2$ (1 mmol/l), $CaCl_2$ (0.6 mmol/l) and glucose (10 mmol/l) (test buffer) at 37 °C. $O_2^{\cdot -}$ radical formation was determined photometrically (UVIKON 820; Kontron, Hannover, Germany) at 550 nm by measuring the reduction of cytochrome *c* (50 μmol/l) [28]. By addition of superoxide dismutase (100 nmol/l) it was confirmed that the reduction of cytochrome *c* was caused by $O_2^{\cdot -}$.

Localization of the superoxide and hydrogen-peroxide-generating system

In order to determine the ultrastructural localization of the superoxide-generating system, the stimulated fibroblasts were incubated with Ce^{3+} which precipitates as cerium peroxide [$Ce(OH)_2OOH$] if the cells generate H_2O_2, or with NBT which is reduced to the insoluble Blue Formazan by generation of $O_2^{\cdot -}$ [29,30].

The cells were cultured on plastic slides as described and washed with Tris/maleate buffer (0.1 mol/l, pH 7.2) containing sucrose (0.15 mol/l) at 37 °C. Half of the slides were pre-incubated for 10 min at 37 °C in the same buffer to which was added 7% sucrose and 1 mmol of 3-amino-1,2,4-triazole/l, as inhibitor of the catalase. The adherent fibroblasts on cover slides were incubated with various stimulants for between 30 min and 4 h in Tris/maleate (0.1 mol/l, pH 7.5) containing sucrose (0.15 mol/l), glucose (10 mmol/l), 3-amino-1,2,4-triazole (10 mmol/l), $CeCl_3$ (1 mmol/l) and NADPH (0.71 mmol/l). The solution was filtered through a 0.2 μm filter to remove cerium hydroxide. The other half of the slides were not pre-incubated but were directly stained with NBT (100 μmol/l) in the test buffer described above until insoluble Blue Formazan was observed. Afterwards the slides were routinely prepared for scanning electron microscopy, including critical point drying and coating with gold.

RESULTS

Determination of a low-potential cytochrome b_{558}

In order to investigate whether a low-potential cytochrome *b*, like that in the neutrophil NADPH oxidase system, is present in fibroblasts, the membranes (20 mg of protein/ml) were set to a defined redox potential as described and spectra were recorded. The midpoint potential was calculated using the Nernst equation with $n = 1$ and $\log(\text{cytochrome } b^{3+}/\text{cytochrome } b^{2+}) = 0$. A midpoint potential ($E_{m,7.0}$) of -243 ± 8 mV was determined. No differences were observable between membranes obtained after prestimulation with interleukin-1 (100 nmol/l) and unstimulated membranes. The cytochrome was rapidly oxidized by oxygen and the addition of azide (1 mmol/l) did not prevent oxidation.

The absolute amount of low-potential cytochrome b_{558} in fibroblast membranes was 10 pmol/mg of protein (Fig. 1). Thus human fibroblasts possess only 10% of the amount of cytochrome b_{558} that is present in human neutrophils (100 pmol/mg).

Inhibition of superoxide formation by DPI

DPI caused rapid inhibition (within the mixing time) of superoxide release in fibroblasts upon stimulation with the following agents: calcium ionophore A23187 (5 μmol/l), interleukin-1 (100 nmol/l), tumour necrosis factor (100 nmol/l),

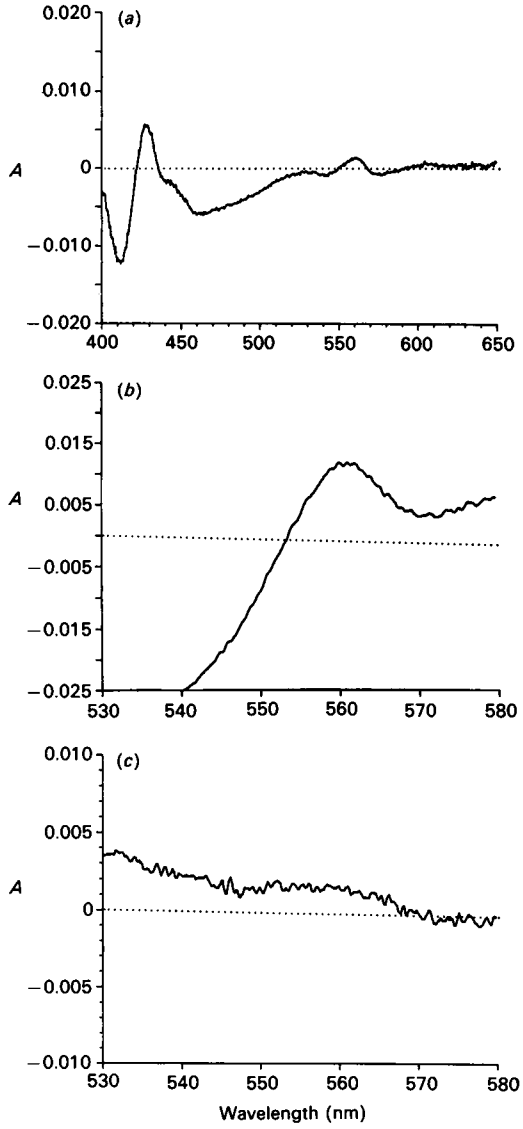

Fig. 1. Reduced-minus-oxidized difference spectra of human fibroblast membranes

Reduced-minus-oxidized difference spectra and spectra at a known redox potential were recorded as described in the Materials and methods section. (*a*) Difference spectrum of fibroblast membranes (dithionite-reduced minus oxidized). The protein content was 0.88 mg/ml. The α-band of the low-potential cytochrome *b* is located at 558 nm, the β-band at 528 nm and the γ-band at 424 nm. (*b*) Quantitative determination of total cytochrome *b* at fixed potential. Difference spectra of the membranes (4.4 g of protein/l) were recorded at −334 mV (completely reduced cytochromes) and +178 mV (completely oxidized cytochromes). The amount of total cytochrome *b* was calculated by measuring the absorption at 559 nm, using $\epsilon_{559-540} = 21.6\ mM^{-1} \cdot cm^{-1}$. (*c*) Quantitative determination of low-potential cytochrome *b* at fixed potential. Difference spectra of the membranes (4.4 g of protein/l) were recorded at −334 mV (completely reduced cytochromes) and −160 mV (completely oxidized low-potential cytochrome *b*, but all other cytochromes reduced). The amount of low-potential cytochrome *b* was calculated by measuring the absorption at 559 nm, using $\epsilon_{559-540} = 21.6\ mM \cdot cm^{-1}$.

PMA (2.5 μmol/l), serum-treated zymosan (1 mg/ml), and joint fluids obtained from patients suffering from rheumatoid arthritis (5%, v/v) [5]. Complete inhibition was obtained, independent of the stimulus (Fig. 2). The fibroblasts are slightly more sensitive to inhibition with DPI than are neutrophils [22].

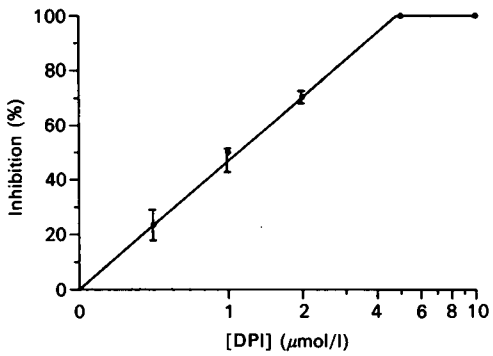

Fig. 2. Dose-dependence of inhibition by DPI of $O^{2\cdot-}$ release by fibroblasts

Ca^{2+} ionophore A23187 (5 μmol/l) was added and the $O_2^{\cdot-}$ was determined. At 2 min after $O_2^{\cdot-}$ release was observable, DPI at different concentrations was added and the initial inhibition was calculated. Data represent means ± S.D. of eight different experiments.

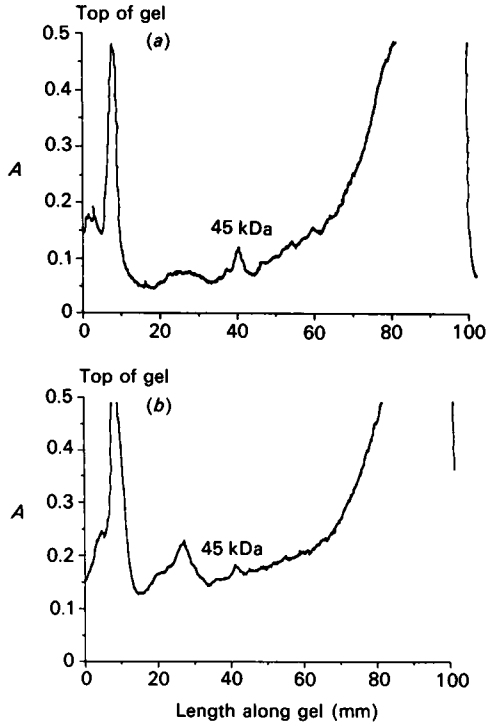

Fig. 3. Labelling of polypeptides by [^{125}I]DPI binding

Labelling of polypeptides and autoradiography were performed as described in the Materials and methods section. [^{125}I]DPI labelling of total homogenates of (*a*) B-lymphocytes and (*b*) fibroblasts is shown.

Determination of a DPI-binding flavoprotein

[^{125}I]DPI binds to a protein of 45 kDa present in fibroblast membranes, similar to a flavoprotein component of the NADPH oxidase system in neutrophils or B-lymphocytes [22–24]. Like the low-potential cytochrome *b*, this 45 kDa protein is also present in lower concentrations in fibroblasts compared with phagocytes and B-lymphocytes (Fig. 3).

Localization of the superoxide- and hydrogen-peroxide-forming system

In order to determine the ultrastructural localization of the

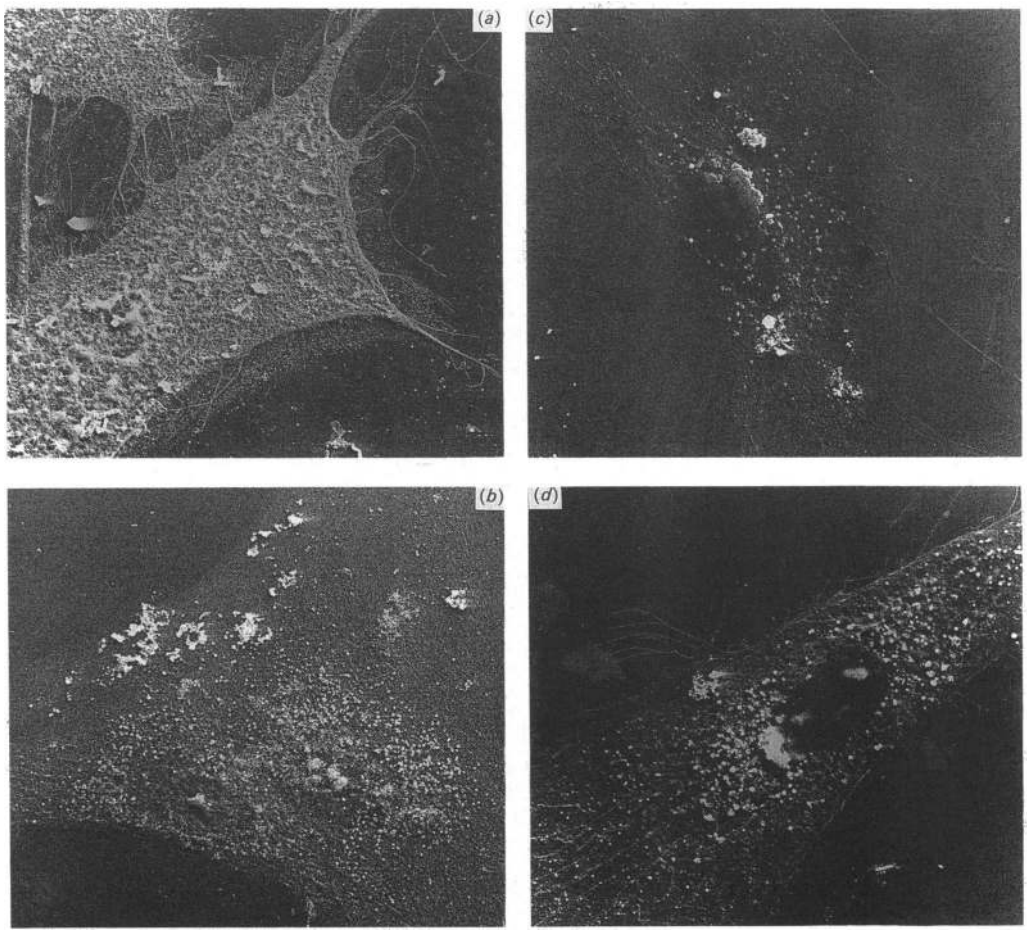

Fig. 4. Localization of H_2O_2 formation by scanning electron microscopy

Peroxide production by adherent human fibroblasts was visualized as described in the Materials and methods section. Cells were (*a*) stimulated with interleukin-1 (100 nmol/l) in the presence of cerium; (*b*) unstimulated in the presence of cerium; (*c*) cerium chloride omitted but stimulated with interleukin-1 (100 nmol/l); and (*d*) not treated. Magnification × 1250.

superoxide- and hydrogen-peroxide-forming system, fibroblasts stimulated with various agents were incubated with NBT or $CeCl_3$. Ce^{3+} reacts with hydrogen peroxide to give an insoluble cerium peroxide [$Ce(OH)_2OOH$] and $Ce(OH)_3OOH$. The precipitate is electron-dense and is unaffected by the solvents used to prepare tissues for electron microscope examination. NBT is reduced to the insoluble Blue Formazan by superoxide radicals. The precipitate has a lower electron density than cerium, but is also observable by light microscopy.

$O_2^{\cdot-}$ and H_2O_2 are released by fibroblasts upon stimulation with various agents, including interleukin-1 (100 nmol/l), tumour necrosis factor (100 nmol/l), Ca^{2+} ionophore A23187 (5 μmol/l), PMA (2.5 μmol/l), serum-treated zymosan (1 g/l) and synovial fluids obtained from patients suffering from arthritis (5%, v/v). This was visualized by the formation of the insoluble Blue Formazan (results not shown) or cerium peroxide, and the ultrastructural localization of the product was determined by scanning electron microscopy (Fig. 4).

In both cases, the stimulated fibroblasts showed rather homogeneous dense precipitates over the plasma membranes, which could not be correlated with any specific cellular compartment. The formation of NBT or $Ce(OH)_2OOH$ was not observed in unstimulated fibroblasts, but, depending on the cell cycle, fibroblasts also release low amounts of superoxide spontaneously (B. Meier, unpublished work).

DISCUSSION

Generation of superoxide by human fibroblasts is rather evenly associated with the cell membrane and might occur by activation of a membrane-bound NADPH oxidase system, as in neutrophils. This suggestion is supported by the finding of complete inhibition of $O_2^{\cdot-}$ formation upon the addition of micromolar concentrations of DPI, a compound described as a specific inhibitor of the leucocyte NADPH oxidase system [22–24]. Moreover, a cytochrome b_{558} with a very low midpoint potential of -243 ± 8 mV, capable of reducing oxygen to superoxide, was demonstrated in fibroblast membranes. This compound has similar properties to the low-potential cytochrome b_{-245} which thus far was regarded to be restricted to leucocytes, including eosinophil and neutrophil granulocytes, macrophages and B-lymphocytes [31]. Other cytochromes *b* which are postulated to be responsible for intracellular superoxide release in tissue cells upon contact with xenobiotics possess higher midpoint potentials, of around +10 mV up to −100 mV for the high- and low-potential forms of cytochrome *b* in mitochondria [32] and −12 mV for cytochrome b_5 located in the membranes of the endoplasmic reticulum [33].

However, the concentration of the low-potential cytochrome *b* and the DPI-binding flavoprotein in fibroblasts is only about 10% of that found in phagocytes. This is in accordance with the

lower amounts of superoxide production in fibroblasts in comparison with phagocytes [4,5]. In PMNs the $O_2^{\cdot-}$ release occurred in the form of a short burst lasting some minutes, whereas fibroblasts, endothelial cells and mesangial cells release $O_2^{\cdot-}$ continuously for up to several hours, suggesting important differences between these two systems. Potent stimulants for fibroblasts (e.g. cytokines) are weak stimulants for phagocytes, and potent stimulants for PMNs (e.g. PMA) only cause a minor activation of fibroblasts or have no stimulatory capacity at all, suggesting a specific regulation of both systems. In contrast with phagocytes, fibroblasts are not damaged by the radicals released upon stimulation. Moreover, a spontaneous release of $O_2^{\cdot-}$, depending on the cell cycle, is observable in fibroblasts, and proliferation can be inhibited by DPI or radical scavengers only in distinct states (G_1-phase) (B. Meier, unpublished work). Superoxide radicals were shown to be involved in the proliferation [34] and chemotaxis [35] of fibroblasts.

Thus the NADPH oxidase in fibroblasts, which possesses strong similarity to the NADPH oxidase in PMNs, has a physiologically different, possibly regulatory, function. It has yet to be determined whether both systems are structurally identical, or only possess high functional similarity.

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