

In situ detection of constitutive superoxide anion production in granules of mast cells

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Summary

3,3'-Diaminobenzidine, in the presence of manganese and cobalt ions, was applied for the detection of superoxide anions in unfixed cryostat sections of rat oesophagus, trachea, skin and intact mesenterium. In all connective tissues, a blue final reaction product was found in a granular form in mast cells. The amount of final reaction product formed after incubation with diaminobenzidine and cobalt ions was increased by the addition of manganese ions. Electron microscopical analysis revealed that the electron-dense final reaction product was exclusively present in the granules of mast cells and on elastin fibres. It was found that the constitutive spontaneous formation of final reaction product in mast cells was enzymatic and dependent on the presence of oxygen in the medium. Of all the enzyme inhibitors and free radical scavengers tested, only azide strongly reduced the amount of final reaction product. It was concluded that the reaction was partly caused by peroxidase activity, but that superoxide anions are also constitutively and spontaneously produced in mast cell granules. The exact enzymatic source could not be established. Whether this property of mast cell granules plays an antimicrobial role in connective tissues can only be speculated.

Introduction

Reactive oxygen species (ROS), which include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid, singlet oxygen ($O_2^1\sim G$) and hydroxyl radicals ($OH\cdot$), play important roles in physiological and pathological processes (for reviews, see Halliwell *et al.*, 1992; Cheeseman & Slater, 1993). *In situ* detection of the formation of ROS in tissues and cells may provide relevant new insights as to their functions in (patho)physiological processes. Recently, Karnovsky (1994) reviewed the methodology developed for the detection of ROS using the activated polymorphonuclear leucocyte (PMN) as the paradigm of a cell, which vigorously generates ROS after activation of NADPH oxidase. Methods for the specific cytochemical localization of H_2O_2 and the H_2O_2 -myeloperoxidase-halide system have existed for many years. Furthermore, a procedure was developed by Karnovsky's research group to detect $O_2^{\cdot-}$ formation by NADPH oxidase (Briggs *et al.*, 1986). The specificity of the reaction is based on the fact that $O_2^{\cdot-}$ oxidizes manganese ions Mn^{2+} to Mn^{3+} , and Mn^{3+} oxidizes diaminobenzidine (DAB).

Therefore, a medium containing DAB- Mn^{2+} was used to localize sites of $O_2^{\cdot-}$ production in stimulated PMNs. Steinbeck *et al.* (1994) applied this technique to demonstrate *in situ* production of $O_2^{\cdot-}$ in osteoclasts actively resorbing bone in intact tissue sections. In our laboratory, the sensitivity of the histochemical method was improved by adding cobalt ions to the DAB- Mn^{2+} -containing incubation medium (Kerver *et al.*, 1997). Final reaction product was found in the mitochondria of parenchymal cells of rat liver and epithelial cells of rat small intestine. It was concluded that superoxide anions and singlet oxygen were detected and that the enzymes, NADH-coenzyme Q reductase and aldehyde oxidase, were responsible for this formation of ROS. Final reaction product was also observed in large cells in the connective tissue of large portal tracts in rat liver (unpublished results). We assumed that these cells were mast cells and we decided to investigate further the formation of final reaction product in mast cells in various connective tissues.

Materials and methods

Male Wistar rats (TNO, Zeist, The Netherlands) weighing 200–250 g were used. The animals had free access to a laboratory diet and tap water and were housed under constant environmental conditions. The animals were killed under ether anaesthesia. Oesophagus, trachea and skin were removed after sacrifice and small fragments up to 5-mm thick were frozen in liquid nitrogen and stored at -80°C until further use (Van Noorden & Frederiks, 1992). Mesenterium was also removed and was stretched over Perspex rings. Cryostat sections (8 μm thick) were cut on a Bright motor-driven cryostat with automatic speed control to ensure constant section thickness at a cabinet temperature of -25°C . The sections were picked up onto clean glass slides and stored until further use. Before incubation, sections were air dried for 5 min at room temperature.

Cryostat sections and intact mesenterium were incubated in a medium containing 12.5 mM DAB (Fluka Chemika, Buchs, Switzerland), 0.0 or 2.5 mM MnCl_2 (Sigma, St Louis, MO, USA) and 0 or 40 mM CoCl_2 (Merck, Darmstadt, Germany) in 10% w/v polyvinyl alcohol (PVA; weight average M_r 70 000–100 000; Sigma) in 100 mM Tris-maleate buffer (pH 8.0) for 30 min at 37°C . After incubation, sections and mesenterium were washed in hot distilled water (60°C) to stop the reaction immediately and to remove the viscous incubation medium. Sections were mounted in glycerol jelly. Mesenterium was first stretched over a glass slide and then mounted in glycerol jelly.

To investigate whether the formation of final reaction product was enzyme mediated, the following experiments were performed: (1) sections were heated for 15 min in distilled water at 37°C , 65°C and 80°C before the incubation; (2) sections were fixed for 5 min in 4% w/v formaldehyde or 0.25% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, before the incubation; and (3) incubation times were varied (15–60 min). The effect of the omission of PVA from the incubation medium was tested to investigate whether the enzyme was tightly bound.

Characterization of the enzymes responsible for the formation of final reaction product was performed by adding the following more or less specific inhibitors to the incubation medium: 10 mM 3-amino-1,2,4-triazole (Serva, Heidelberg, Germany) for catalase, 1 or 5 mM KCN (Merck) for catalase, peroxidase, cytochrome *c* oxidase, superoxide dismutase, 5 mM *N*-ethylmaleimide (BDH, Poole, UK), blocker of SH groups, 10 mM 4-hydroxymercuribenzoic acid (Serva), blocker of SH groups, 0.5 mM $(\text{NH}_4)_2\text{S}$ for cytochrome *c* oxidase, 1 mM allopurinol (Sigma) for xanthine oxidase, 10 mM aminoguanidine (Sigma) for diamine oxidase, 2.5 mM NAD^+ (Boehringer, Mannheim, Germany) for NADH oxidase, 2.5 mM NADP^+ (Boehringer) for NADPH oxidase, 0.5 mM diphenyl iodonium (Aldrich, Brussels, Belgium) for NADPH oxidase, 0.01 mM rotenone (Merck) or 0.1% w/v Triton X-100 (Merck) for NADH - coenzyme Q reductase and 5 mM menadione (Sigma) or 0.5 mM disulfiram (Sigma) for aldehyde oxidase (Zollner, 1989). The involvement of oxygen in the reaction was studied by incubating sections in media saturated with nitrogen, pure oxygen and air using a tonometer (Van Noorden & Frederiks, 1992). The

type of ROS that was generated after incubation was established by using the following more or less specific ROS quenchers or scavengers: 5–5000 units catalase (from bovine liver, Sigma), 100–1000 units superoxide dismutase (SOD, from bovine erythrocytes; Boehringer), 5–100 mM NaN_3 (Merck), 5 mM histidine (Merck), 5 mM benzoquinone (Sigma), 5 mM TEMPO (Sigma), or 5 mM mannitol (Janssen Chimica, Beerse, Belgium).

The ultrastructural localization of final reaction product was studied in mesenterium after being fixed in 0.25% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 5 min at room temperature followed by incubation in the DAB- Mn^{2+} - Co^{2+} -containing medium for 30 min at 37°C . After incubation, mesenterium was post-fixed in 1% OsO_4 in 100 mM sodium cacodylate buffer, pH 7.4, for 1 h at room temperature, dehydrated and embedded in Epon resin LX112 according to routine procedures. Ultrathin sections were used either unstained or stained with Uranyl Acetate and Lead Citrate and studied with a Zeiss EM 10c transmission electron microscope.

Results

Final reaction product was generated as a blue DAB polymer complexed with cobalt in epithelial cells and smooth and striated muscle cells of oesophagus, skin and trachea after incubation of unfixed cryostat sections in a medium containing DAB, MnCl_2 and CoCl_2 in 10% PVA. Moreover, final reaction product was found in the large cells of connective tissue beneath the epithelial cells (Fig. 1). In mesenterium, the granules of mast cells contained high amounts of final reaction product (Fig. 2). Weak staining was present on elastin fibres (Fig. 2). When only incu-

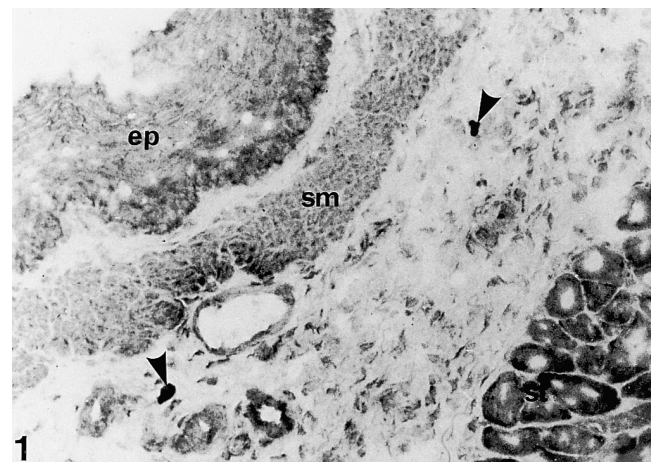


Fig. 1. Photomicrograph of a cryostat section of rat oesophagus incubated with a DAB- Mn^{2+} - Co^{2+} -containing medium. Small amounts of final reaction product are present in epithelial cells (ep), smooth (sm) and striated (st) muscle cells and in cells of connective tissue. Large amounts are present in mast cells of connective tissue (arrowhead) ($\times 165$).

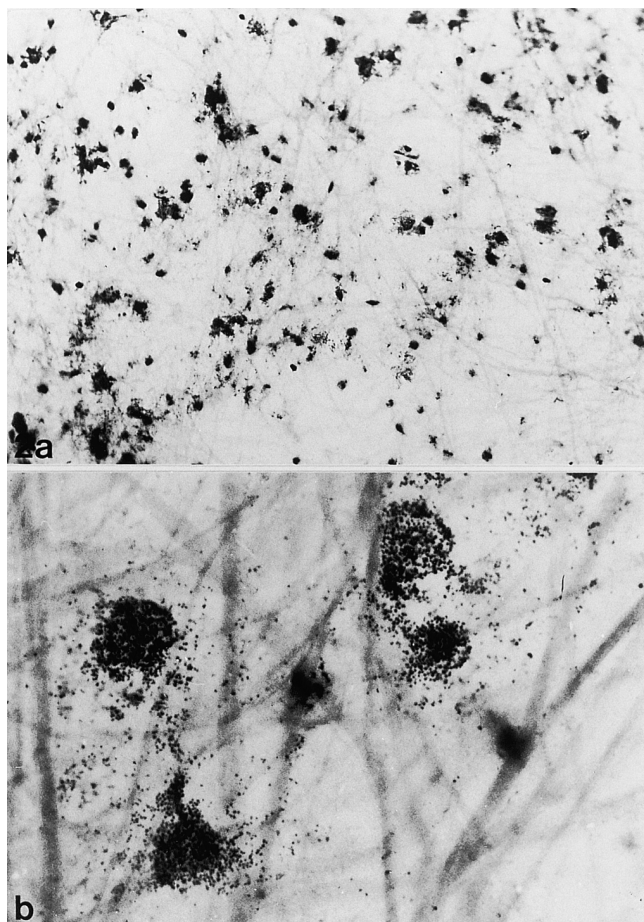


Fig. 2. Photomicrographs of mesenterium of the rat incubated with a DAB-Mn²⁺-Co²⁺-containing medium. Large amounts of final reaction product are present in the granules of mast cells. A weak staining is present on elastin fibres. Many mast cells have been degranulated. (A) $\times 82$; (B) $\times 330$.

bating in DAB, no staining was found. DAB in the presence of manganese also did not give rise to any staining. Addition of cobalt ions to DAB enhanced the staining intensity of mast cells and elastin fibres, but the reaction was further intensified by addition of manganese ions to the Co-DAB medium. The presence of high amounts of final reaction product in the granules of mast cells and on elastin fibres was confirmed by electron microscopy (Fig. 3). It was concluded that mast cells in all tissues tested showed similar characteristics with respect to the staining with the DAB-Mn²⁺-Co²⁺-containing incubation medium. Therefore, the characteristics of the system generating final reaction product in mast cells were only studied in the oesophagus. Formation of final reaction product because of Co²⁺, Mn²⁺ and DAB was not affected by preincubation of the sections in an aqueous medium at 37°C, but preincubation at 65°C or 80°C resulted in complete inhibition of final

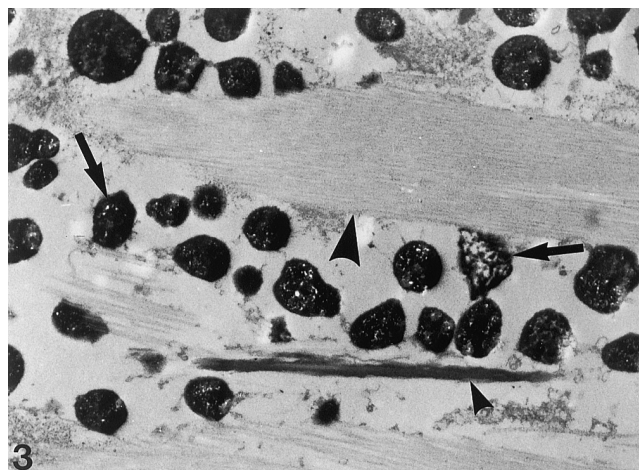


Fig. 3. Electron micrograph of mesenterium of the rat incubated with a DAB-Mn²⁺-Co²⁺-containing medium. Electron-dense precipitate is present in the granules of mast cells (arrow) and on elastin fibres (small arrowhead). Collagen fibres are devoid of precipitate (large arrowhead). Ultrathin sections were not stained with Uranyl Acetate and Lead Citrate ($\times 23120$).

reaction product formation. Omitting PVA from the incubation medium did not affect the staining intensity. Prefixation of the sections in 4% formaldehyde abolished formation of final reaction product completely, but it was not affected by prefixation in 0.25% glutaraldehyde. The amount of final reaction product increased with increasing incubation times from 15 to 60 min. It was concluded from these results that it is likely that the activity of one or more tightly bound enzymes was responsible for the formation of precipitate in the mast cell granules. The involvement of oxygen as substrate in the reaction was proved because sections that were incubated in the presence of 100% oxygen contained final reaction product, whereas final reaction product was not formed after incubation in 100% nitrogen. There was no difference between the amounts of final reaction product generated after incubation in the presence of 100% oxygen or air, indicating that the oxygen concentration was not rate limiting. Therefore, all further experiments were performed in air.

The effects of the addition of enzyme inhibitors to the incubation media on the amount of final reaction product formed by granules in mast cells were all negligible. This lack of inhibitory effects means that peroxisomal catalase, mitochondrial cytochrome *c* oxidase and NADH - coenzyme Q reductase, cytoplasmic xanthine oxidase, diamine oxidase, aldehyde oxidase and plasma membrane-bound NADPH oxidase were not involved. Furthermore, the responsible enzyme(s) did not contain essential SH-groups in the active site.

The results of incubations of sections in the presence of ROS quenchers were as follows. Exogenous catalase, which uses H_2O_2 as substrate, mannitol that prevents the formation of OH^\cdot , exogenous SOD, which uses $O_2^{\cdot-}$ as substrate with concomitant formation of H_2O_2 , benzoquinone and TEMPO, specific scavengers of free radicals, and histidine, a scavenger of $O_2^{\cdot-}$, all did not affect the staining intensity in mast cells. Only the addition of azide to the incubation medium reduced the amount of precipitate formed; the extent of inhibition was related to the azide concentration. Azide is an inhibitor of many enzymes, such as those of the respiratory chain in mitochondria, but may also act as a scavenger of superoxide anions.

Discussion

The present study shows that mast cell granules as well as elastin fibres in various connective tissues of rats produce ROS during incubation in a DAB-Mn²⁺-Co²⁺-containing medium (Figs 1 and 2). We venture to suggest that superoxide anions were produced by mast cell granules because the reaction was dependent on the presence of oxygen, the reaction did not occur in the absence of Mn⁺ or Co²⁺ in the DAB-containing media and azide inhibited the reaction strongly. The lack of any inhibiting effect of SOD on the formation of precipitate may be caused by limited penetration of this large enzyme molecule into the tissue (Frederiks *et al.*, 1987; Frederiks & Bosch, 1996). Part of the reaction may result from peroxidase activity because, in the absence of manganese ions, some staining was observed.

Oxygen radicals in mast cells are probably produced by an enzyme because the reaction was sensitive to fixation and heat, and the amounts of final reaction product increased with prolonged periods of incubation. The enzyme(s) must be tightly bound because omission of PVA from the incubation medium did not significantly affect the reaction. No definite conclusions can be drawn with respect to the type of enzyme(s), which is (are) responsible for the production of superoxide anions. All enzyme inhibitors investigated did not exert any effect. The properties of the system responsible for the formation of final reaction product on elastin fibres has not been investigated in the present study. This will be part of a future study on the significance of oxygen radical-related processes in the extracellular matrix. It was recently found that high amounts of extracellular superoxide dismutase were present in the extracellular matrix (Oury *et al.*, 1994, 1996), as well as high enzyme activity (Frederiks & Bosch, 1997).

The significance of the constitutive production of

$O_2^{\cdot-}$ in granules of mast cells in connective tissues remains intriguing, but unknown. It has been demonstrated that superoxide anion production can trigger cell division (Murrell *et al.*, 1989) and serves as a physiological regulator of this process. Under other circumstances, production of oxygen radicals induces apoptosis (Rothstein *et al.*, 1994). Under normal conditions, superoxide anions are converted into hydrogen peroxide by superoxide dismutase (Fridovich, 1978). It is assumed that there is a balance between superoxide production and superoxide scavenging under normal conditions (McCord, 1995). Indeed, mast cell granules do not only contain superoxide dismutase but also catalase and peroxidase (Gruber & Kaplan, 1989). It can be hypothesized that mast cells may act as activated neutrophils by disturbing the balance between the formation and scavenging of oxygen radicals and exert bactericidal activity in that way.

In conclusion, mast cell granules were found to produce oxygen radicals enzymatically, but the type of enzyme responsible for this phenomenon and the significance of this constitutive production need to be investigated further.

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