Review

Cytochemistry of NADPH Oxidase Activity in Human Neutrophils

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Human neutrophils respond with a potent and sustained production of superoxide by NADPH oxidase activity after exposure to various stimulants. Superoxide is then converted to other reactive oxygen species. These oxidants participate in microbial killing, cause injury to surrounding tissues, regulate apoptosis of neutrophils, and, further, are viewed as a signal transduction messenger. We present herein the cytochemical approach for detecting NADPH oxidase activity in human neutrophils under both an electron microscope and a CCD camera-equipped

fluorescence microscope. The applications of the cytochemical methodology are also mentioned in this review. Especially, the following mechanisms for the release of superoxide from human neutrophils are considered: the active NADPH oxidase is located exclusively in intracellular compartments which are delivered to the plasma membrane by membrane fusion events, and the vesicles whose membrane contain the active oxidase cycle between the interior of the cell and the plasma membrane.

Key words: Cytochemistry, NADPH oxidase, Neutrophils, Superoxide

I. Introduction

Human neutrophils possess an enzymatic complex, the NADPH oxidase, which catalyzes the one-electron reduction of molecular oxygen to superoxide (O_2^-). Organization of NADPH oxidase is associated with several components. Upon stimulation, duo-phox cytosolic components of NADPH oxidase (p40-phox and p47-phox) bind to another cytosolic component (p67-phox) which is associated with actin filaments [6]. Thus, the tri-phox cytosolic components of NADPH oxidase (p40-phox, p47-phox and p67-phox) come to bind to actin filaments. Activated protein kinase C (PKC), translocated from the plasma membrane, phosphorylates the cytosolic components at a scaffold of cytoskeleton. Subsequently, p40-phox, responsible for maintaining the resting state of NADPH oxidase, is separated from other two cytosolic phox proteins following an attachment of the active form of small GTP-binding protein Rac to p67-phox. The cytosolic duo-phox proteins (p47-phox and p67-phox) then conjugate with the membrane components (cytochrome b_{558}

consisting of a p22-phox and a gp91-phox). This chain of events triggers production of O_2^- [13]. The formed O_2^- generated from the NADPH oxidase is converted to other reactive oxygen, including hydrogen peroxide, hydroxyl radical, singlet oxygen and hypochlorous acid. These oxidants participate in microbial killing, cause injury to surrounding tissues and regulate apoptosis of neutrophils [7]. The aim of this review is to provide the cytochemical methodology for detection of oxidant-producing sites in human neutrophils by re-evaluating the cytochemical data available.

II. Assay of NADPH Oxidase Activity under Electron Microscope

In situ localization of enzyme activity in cells and tissues can be achieved by enzyme cytochemical and histochemical analyses. Several cytochemical methods have been reviewed to detect NADPH oxidase activity [10, 21]. Two distinct types of methods, the cerium-based method and diaminobenzidine (DAB)/ Mn^{2+} -based method, have been mainly employed to detect the localization of NADPH oxidase activity in human neutrophils under the electron microscope. The cerium method has contributed towards the visualization of the NADPH ox-

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idase-activating sites in cells [3]. The enzymatic reaction is as follows: O_2^- generated by NADPH oxidase activity is dismutated to form hydrogen peroxide (H_2O_2). Cerium ion (Ce^{3+}) reacts with H_2O_2 to form cerium hydroxide ($Ce(OH)_2OOH$) which is the electron-dense precipitate observed under the electron microscope. The DAB/ Mn^{2+} method was originally developed to detect O_2^- -producing sites in neutrophils [4, 24, 25]. The enzymatic reaction is as follows: DAB is oxidized by Mn^{3+} which is formed from Mn^{2+} by the oxidation with O_2^- . The oxidized DAB then reacts with osmium ions to form electron-dense insoluble polymers detectable under the electron microscope.

Human neutrophils contain a heterogeneous population of intracellular granules, which have been classified into five distinct types: (i) defensin-positive azurophil granules; (ii) defensin-negative azurophil granules; (iii) specific granules; (iv) gelatinase granules; and (v) secretory vesicles [1]. Recently, Price *et al.* [20] discovered a novel vesicle, containing inhibitor of matrix metalloproteinases-1, that does not fuse with the phagosome in human neutrophils. Among these intracellular compartments, studies have shown that cytochrome b_{558} is detectable in membranes of specific and gelatinase granules and secretory vesicles [1]. It should be noted, however, that cell surface expression of cytochrome b_{558} is mostly caused by the association of the secretory vesicles with the plasma membrane, since the secretory vesicles were reported to mobilize rapidly compared to other types of granules upon stimulation [5].

The previous experimental view of O_2^- release in

human neutrophils is that the various components of the NADPH oxidase complex are brought into juxtaposition at the plasma membrane upon appropriate stimulation. Using the DAB/ Mn^{2+} method mentioned above, we studied the localization of the oxidant-producing sites in human neutrophils stimulated with a protein kinase C activator, PMA. In our experiments, O_2^- produced by the NADPH oxidase was detected in an intracellular compartment. Originating as intracellular granules, these oxidant-producing compartments fuse directly with the plasma membrane or fuse with other intracellular structures, including endocytic vesicles, which in turn can fuse with the plasma membrane. This pathway of exocytosis was predicted for the following reasons: (i) rod-shaped granules are the minimum structural elements among the oxidant reaction-positive compartments; (ii) longer term stimulation induces a decrease in the number of compartments containing only the oxidant reaction-positive materials and an increase in the number of structures containing both the oxidant-reaction product and ferritin particles that are used as an endocytic marker; (iii) the O_2^- -positive rod-shaped granules can fuse with endocytic compartments; and (iv) the stimulated cells exhibit exocytosis of oxidant-positive granules. In the latter case, tracer studies demonstrated that the limiting membrane of the oxidant-producing granules was not derived from the plasma membrane [12]. These structures are possibly generated by Golgi complexes [11]. In addition, we tried to identify the oxidant-producing intracellular compartments, among a heterogeneous population of granules, using an intracellularly localized enzyme activity, alkaline

Fig. 1. Fluorescence micrographs of living human neutrophils. Cells were exposed to succinimidyl ester of dichlorodihydrofluorescein diacetate (H_2DCFDA) followed by stimulation with phorbol myristate acetate. (A) Image of cells isolated from healthy volunteers. Fluorescence is observed in intracellular compartments of these cells. (B) Image of cells isolated from patients with chronic granulomatous disease lacking gp91-phox, a membrane component of NADPH oxidase. No fluorescence is visualized in intracellular compartments of these cells. Bars = 10 μm .

phosphatase (ALPase), which is found in a unique compartment that is distinct from the azurophil and specific granules [11]. The finding that ALPase activity and the

oxidant reaction are co-localized in the same compartments confirmed that the ALPase-containing compartments are the site of oxidant production prior to its

Fig. 2. Fluorescence micrographs showing oxidant-producing intracellular compartments (green color in **A**), and endocytic vesicles (red color in **B**). H_2DCFDA -loaded cells were exposed to TRITC-dextran as an endocytic marker. Images **A** and **B** were superimposed to detect structures (arrow heads) formed by fusion of oxidant-producing intracellular compartments (arrows) and endocytic vesicles (double arrows) (**C**). Bars = 10 μm .

release from PMA-stimulated neutrophils [12]. Thus, the oxidant-producing intracellular compartments correspond with secretory vesicles which include both ALPase and cytochrome b_{558} of NADPH oxidase [2]. We reported previously that the ALPase-positive granules can associate with the phagosomes [14]. The oxidant-producing organelles do not seem to include the novel vesicles discovered recently by Price *et al.* [20], since these structures are incapable of fusing with the phagosomes [20].

III. Assay of NADPH Oxidase Activity under Fluorescence Microscope

Recently, we developed a simple methodological approach to evaluate the intracellular movement of the oxidant-producing compartments in living human neutrophils loaded with the succinimidyl ester of dichlorodihydrofluorescein diacetate (H_2DCFDA), which is not fluorescent until oxidized by reactive oxygen species [16]. This assay system is as follows: H_2DCFDA -loaded cells are placed onto a poly-D-lysine-coated glass coverslip at the bottom of a microwell dish. Then, prewarmed ($37^\circ C$) PBS is added to the dish and the latter is located on the thermoregulating stage of a fluorescent microscope to ensure constant temperature at $37^\circ C$. The microscope is equipped with a cooled CCD camera, a 100-W mercury lamp, and appropriate sets of filters. We used low light conditions (5% of maximum light intensity) due to the high susceptibility of H_2DCFDA to photo-oxidation under standard conditions used in fluorescence microscopy [22].

Using the method mentioned above, when H_2DCFDA -loaded cells isolated from healthy volunteers are stimulated with PMA, fluorescence was visualized in intracellular compartments (Fig. 1A). As a control, we examined neutrophils isolated from patients with X-linked chronic granulomatous disease lacking gp91-phox, a membrane component of NADPH oxidase. No fluorescence was detected in intracellular compartments of these cells even upon stimulation with PMA (Fig. 1B). This finding indicates that fluorescence detected in stimulated cells is attributed solely to oxidants generated by NADPH oxidase. Experiments with NADPH oxidase-deficient neutrophils, thus, confirm that the assay system mentioned above does not detect fluorescence generated from other organelles than the specialized oxidant-producing intracellular compartments, such as mitochondria, which are known to produce the reactive oxygen species [9, 17]. In this point of view, the signal of the oxidants generated by mitochondria is probably too weak to be detected by the mentioned assay system. Applying this methodological approach, we can perform a real-time detection of fluorescence generated by oxidant-producing intracellular compartments, and also enable tracking of the movement of a single oxidant-producing intracellular compartment following cell stimulation. Importantly, when cells are exposed to H_2DCFDA followed by loading with TRITC-dextran which is a marker of endocytic compartments, we

can simultaneously detect oxidant-producing intracellular compartments, endocytic vesicles, and their fused structures (Fig. 2A–C). The significance of this finding is in the simple observation of the pattern of fusion of oxidant-producing intracellular compartments with the plasma membrane-derived endocytic vesicles.

IV. Conclusion

The cytochemical study using electron microscope defined the precise localization of NADPH oxidase activity in human neutrophils. In addition, we mentioned herein that the methodological approach using H_2DCFDA as an oxidant-detecting reagent facilitates visualizing the oxidant-producing intracellular compartments, tracing the intracellular movement of them, and observing the structures formed by fusion of oxidant-producing intracellular compartments with endocytic vesicles in living human neutrophils.

Function of the cytoskeleton associates with structures involved in the activation of NADPH oxidase [19] and exocytosis of oxidant-producing intracellular compartments [15]. For instance, ML-7, an inhibitor of myosin light chain kinase (MLCK), associating with actomyosin assembly and contraction [23], was reported to inhibit O_2^- production by human neutrophils [8]. MLCK is activated by the upstream effectors including extracellular signal-regulated protein kinase (ERK), mitogen-activated protein kinase kinase (MEK) and PKC [18]. This signaling pathway remains to be established. Moreover, the oxidants generated by neutrophils were reported to participate in apoptosis of the cells through the inhibition of caspase activity [7]. The precise signaling pathways, however, are not known yet. We are, therefore, convinced that the cytochemical approach described in this review is a useful tool to elucidate the signaling pathways controlling both the activation of NADPH oxidase and dynamics of oxidant-producing intracellular compartments.

V. References

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