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An improved superoxide-generating nanodevice for oxidative stress studies in cultured cells

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ABSTRACT

The effects of reactive oxygen species on cells have attracted great attention from both physiological and pathological aspects. Superoxide (O_2^-) is the primary reactive oxygen species formed in animals. We previously developed an O_2^--generating nanodevice consisting of NADPH oxidase 2 (Nox2) and modulated activating factors. However, the device was subsequently found to be unstable in a standard culture medium. Here we improved the device in stability by cross-linking. This new nanodevice, Device II, had a half-life of 3 h at 37 °C in the medium. Device II induced cell death in 80% of HEK293 cells after 24 h of incubation. Superoxide dismutase alone did not diminish the effect of the device, but eliminated the effect when used together with catalase, confirming that the cell death was caused by H_2O_2 derived from O_2^-. Flow cytometric analyses revealed that Device II induced caspase-3 activation in HEK293 cells, suggesting that the cell death proceeded largely through apoptosis.

1. Introduction

There has been increasing interest in the effects of reactive oxygen species (ROS) on cells and tissues. ROS function as bactericidal agents in host defense and as signaling molecules in intracellular signaling pathways for several cell functions [2,5,9,3]. Despite these physiological roles, ROS can be harmful toward cells and their excessive formation can bring about oxidative stress, tissue damage, and other pathological effects [3,10]. Thus, it has become important to clarify how ROS at high concentrations affect cells and tissues.

The primary source of ROS in animals is superoxide (O_2^-) in most cases. The formed O_2^- is readily converted to hydrogen peroxide (H_2O_2) spontaneously or enzymatically. Other ROS such as the hydroxyl radical ($OH^\bullet$) or singlet oxygen ($^1O_2$) are produced by reactions between O_2^- and H_2O_2. O_2^- is also produced through electron leakage from the mitochondrial electron transport chain or as a by-product of certain enzymes including xanthine oxidase, NO synthase, and cytochrome P-450 reductase. In addition, O_2^- is produced by the NADPH oxidase (Nox) family (Nox1, Nox2, Nox3 and Nox5) expressed in several tissues and organs [9,3] although H_2O_2 is produced as the primary product of some Nox enzymes, such as Nox4 [19] and Duox/Duoxa system [16].

Because O_2^- is a highly reactive agent, high levels of O_2^- damage cells and tissues, resulting in disorders. To examine how O_2^- affects cells, a tool that can produce O_2^- constantly at a high rate is required. Although a xanthine/xanthine oxidase system has been used for this purpose, the system has several demerits for cell experiments (see Section 4).

We previously developed an O_2^--generating nanodevice based on Nox2 enzyme [22]. Nox2 was the first discovered Nox family enzyme in phagocytic cells, and subsequently found in many other types of cells. Nox2 activation requires three cytosolic factors $p47^{phox}$, $p67^{phox}$, and Rac (a small GTPase). Although cell-free activation of Nox2 was developed a long time ago [4], no attempts had been made to use it as an O_2^--generator because its activation requires: (i) multiple regulatory proteins; (ii) unmasking of these proteins; (iii) GTP loading on Rac; and (iv) SDS or another anionic amphiphile as a stimulant. Furthermore, its activation is transient.

Abbreviations: ROS, reactive oxygen species; Nox, NADPH oxidase; MEM, Eagle's minimal essential medium; cyt.b_{558}, cytochrome b_{558}; RacQ61L, Rac(Q61L, C189S); p67N–p47N, $p67^{phox}$(1-210)-$p47^{phox}$(1-286); PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(ethanesulfonic acid); EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; sulfo-NHSN, -hydroxysulfosuccinimide; SOD, superoxide dismutase; NV, DEVD-NucView488; PI, propidium iodide.

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We previously improved the cell-free activation method in several points and established an O_2^--generating nanodevice that produced high levels of O_2^- continuously without any stimulant [22]. The nanodevice was composed of cytochrome b_{558} (cyt.b_{558}) (Nox2 + p22^{phox}), a lipid mixture including phosphatidylinositol, and regulatory proteins unmasked by genetic engineering. The device was found to be highly efficient and stable at 37 °C in ordinary buffers such as phosphate and Tris buffers.

However, the nanodevice was subsequently revealed to be unstable in Eagle's minimal essential medium (MEM), a widely used standard culture medium. We tried to identify the medium constituents that inactivated the device, but were unsuccessful. Therefore, we utilized another strategy involving cross-linking, which we had previously used to stabilize the Nox2 complex [21,15]. By optimizing the conditions for cross-linking, we successfully improved the stability of the nanodevice in MEM and named it Device II.

In the present study, we established a preparation method of Device II and confirmed its ability to generate O_2^- under conditions used for cell culture. Using the new nanodevice, we investigated the effect of O_2^- on HEK293 cells. We found that Device II efficiently induced cell death in these cells, in association with caspase-3 activation.

2. Materials and methods

2.1. Materials

Superoxide dismutase (bovine erythrocyte) (SOD), catalase (bovine liver) (C-3155, aqueous solution), cytochrome *c* (horse heart), and MEM (M3024, without phenol red) were purchased from Sigma–Aldrich (St. Louis, MO). MEM (with phenol red, 10370021) and trypsin-EDTA were obtained from Life Technologies (Carlsbad, CA) and fetal bovine serum was from Biowest (Nuaillé, France). Propidium iodide (PI) was purchased from Dojindo (Kumamoto, Japan). DEVD-NucView488 (NV) was obtained from Biotium (Hayward, CA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was purchased from Nacalai Tesque (Kyoto, Japan) and *N*-hydroxysulfosuccinimide (sulfo-NHS) was from Thermo Fisher Scientific (Rockford, IL).

2.2. Preparation of NADPH oxidase components

Modulated cytosolic factors p67^{phox}(1-210)-p47^{phox}(1-286) (p67N-p47N) and Rac(Q61L, C189S) (RacQ61L) were prepared as previously described [14]. Cyt. b_{558} was purified from porcine neutrophils [6], relipidated with several phospholipids including phosphatidylinositol as described [22], and stored at −80 °C.

2.3. Preparation of Device I and Device II

Device I was prepared as described for the original device [22] except for the cyt.b_{558} concentration. The activation mixture contained cyt.b_{558} (1 μM), p67N-p47 N (25 μM), and RacQ61L (25 μM) in buffer A (50 mM PIPES pH 7.0, 8 mM $MgCl_2$, 10 μM FAD), and was incubated for 5 min at 25 °C. The mixture was designated Device I and frozen in aliquots.

Device II was prepared from Device I by the procedure summarized in Fig. 1. Device I was diluted 10-fold with buffer A and kept at 25 °C for 5 min. Subsequently, 10 mM EDC and then 5 mM sulfo-NHS were added slowly to the mixture with gentle stirring. The mixture was allowed to stand at 25 °C for 30 min and stirred every 10 min. The mixture was then dialyzed against 50 mM PIPES buffer (pH 7.0) containing 10 μM FAD and 20% glycerol at 4 °C for 4 h. The mixture was designated Device II and frozen in aliquots.

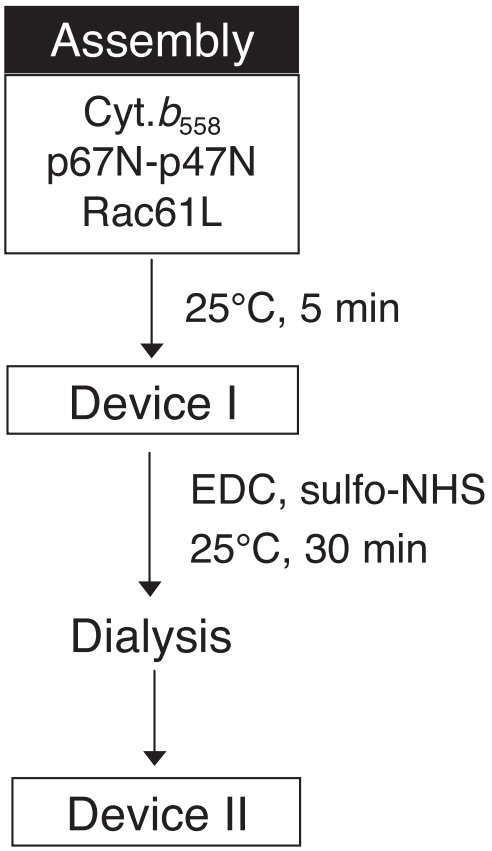

Fig. 1. Procedure for preparation of Device II from Device I.
Cyt.b_{558} (Nox2/p22) was purified and relipidated with phosphatidylinositol and other lipids. The purified cyt.b_{558} was incubated with p67N-p47 N and RacQ61L at 25 °C for 5 min in the presence of FAD (Device I). After 10-fold dilution, the mixture was treated with EDC and then sulfo-NHS, and the mixture was dialyzed (Device II). Other experimental conditions were as described under experimental sections.

2.4. Stability of O_2^--generating activity in MEM

Device I or Device II was diluted 23-fold with MEM (without phenol red) and incubated at 37 °C. At specified time points, aliquots of the mixture (250 μl; containing 1 pmol of cyt.b_{558}) were transferred to the wells of a 96-well microplate containing 200 μM NADPH and 160 μM cytochrome *c*. O_2^- generation was assayed at 25 °C by monitoring cytochrome *c* reduction at 550 nm using a Spectra Classic microplate reader (Tecan, Grodig, Austria) as previously described [13]. The NADPH oxidase activity was expressed as mol O_2^-/min/mol cyt.b_{558}.

2.5. Cell culture

HEK293 cells (Riken Bioresource Center) were maintained in MEM (with phenol red) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C under 5% CO_2 in 96 mm dishes.

2.6. Effect of the nanodevices on viability

HEK293 cells (2×10^5) were seeded into the wells of a 24-well plate containing 800 μl of the above medium, and cultured for 24 h at 37 °C. The cells were then detached from the wells by treatment with trypsin-EDTA, centrifuged for 5 min at 100 × *g*, and suspended in MEM without serum (400 μl). Device II diluted twice with buffer A (total: 16 μl) or undiluted Device II (20 μl) was added to individual wells, corresponding to 0.8 and 2 pmol cyt.b_{558}/well, respectively. O_2^- generation was started by the addition of NADPH