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

Minoru Tamura [a,*], Sachio Kunihiro [a], Yuki Hamashima [a], Yuki Yoshioka [a], Shigenobu Tone [b], Kenji Kameda [c]

[a] Department of Applied Chemistry, Graduate School of Science and Engineering, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan
[b] Deparetment of Biochemistry, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan
[c] INCS, Shigenobu Station, Ehime University, Shitsukawa, Toon, Ehime 791-0295, Japan

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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.

* Corresponding author. Tel.: +81 89 927 9938; fax: +81 89 927 8546.
E-mail address: tamura@ehime-u.ac.jp (M. Tamura).

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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.

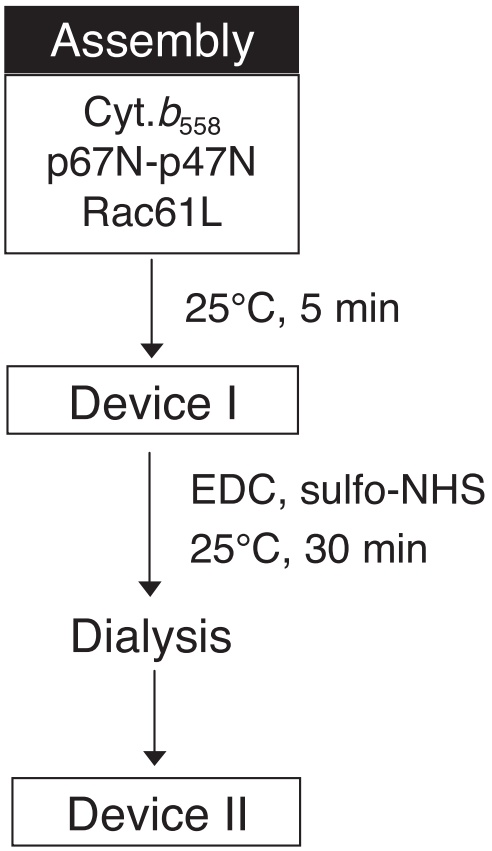

Fig. 1. Procedure for preparation of Device II from Device I.
Cyt.b_{558} (Nox2/p22) was purified and relipidated with phosphatidylinositol and other lipids. The purified cyt.b_{558} was incubated with p67N-p47 N and RacQ61L at 25 °C for 5 min in the presence of FAD (Device I). After 10-fold dilution, 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

(final: 400 μM). After incubation for 24 h at 37 °C, the cells were detached from the wells and processed as described above. The sedimented cells were suspended in phosphate-buffered saline (PBS), stained with trypan blue, and counted.

2.7. Flow cytometric analysis

HEK293 cells were incubated for 24 h at 37 °C in the wells of a 24-well plate containing 400 μl of serum-free medium with or without Device II (0.4 pmol cyt.b_{558} equivalent) plus 400 μM NADPH. The cells were detached from the wells, centrifuged for 5 min at 200 × *g*, washed with PBS, and suspended in the same buffer. The cells were stained with NV (0.2 μM) for 15 min and PI (0.2 μM) for 5 min at 25 °C, and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ).

2.8. Statistical analysis

Statistical comparisons were performed with a two-tailed Student's *t*-test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Stability of Device I in MEM

We previously developed an O_2^--generating device using a highly stabilized Nox2 complex [22], now designated Device I (Fig. 1). The O_2^--generating activity of the device was very stable in regular buffers such as phosphate or Tris-chloride buffers. However, the device was subsequently found to be unstable in culture medium, such as MEM (Fig. 2A). In this medium, the activity decayed rapidly at 37 °C ($t_{1/2}$ = 6 min) for the first 30 min and then decreased slowly (Table 1). The biphasic decay suggested that the preparation was heterogeneous, with only a few stable populations and mostly labile populations.

3.2. Improvement of the stability of Device I

We extensively tried to identify the medium components that destabilized the O_2^--generating activity, but were unsuccessful. Therefore, we tried another approach, *i.e.,* chemical cross-linking, that we previously used to extend the lifetime of the active complex [21,15]. Fig. 1 illustrates the procedure for cross-linking of Device I.

Table 1
Half-lives of the O_2^--generating activity of Device I or Device II in MEM.

Treatment	Name	Half-life (min)[a]	
		1st phase	2nd phase
None	Device I	6 ± 1	124 ± 12
EDC	–	25 ± 4	125 ± 13
EDC + sulfo-NHS	Device II	185 ± 18	–

[a] The activity was measured after incubation at 37 °C in MEM. Half-lives ($t_{1/2}$) were estimated from a first-order plot of the data in Fig. 2 using linear least-squares regression analysis.

When Device I was treated with EDC, the first phase of decay was somewhat improved ($t_{1/2}$ = 25 min) (Table 1). However, the effect was not enough and the decay curve was still biphasic (Fig. 2A). When EDC was used with sulfo-NHS, which is known to stabilize the intermediate of the EDC reaction, the stability was considerably improved, although the initial activity was decreased to one-half compared with Device I. The decay curve became monophasic, and the half-life of the activity was extended to 185 min (Fig. 2A, Table 1). In this system, about 40% of the initial activity was retained, even after incubation for 5 h. We designated the improved version Device II. Fig. 2B showed the actual activities of each sample after the indicated incubation periods. Although Device II had a lower initial activity than others, the activity was more resistant to the incubation at 37 °C in MEM.

3.3. Effect of Device II on HEK293 cells.

The effect of Device II on HEK293 cells was examined and compared with that of Device I. When Device I was added to the cells at 0.8 and 2 pmol cyt.b_{558} equivalent/well, the percentages of surviving cells after 24 h of incubation were 62% and 39% relative to control cells, respectively (Fig. 3). In comparison, when Device II was added at 0.8 and 2 pmol cyt.b_{558} equivalent/well, the percentages of surviving cells were 19% and 12% relative to control cells, respectively. These findings indicated that Device II was more effective in inducing cell death of HEK293 cells than Device I. When

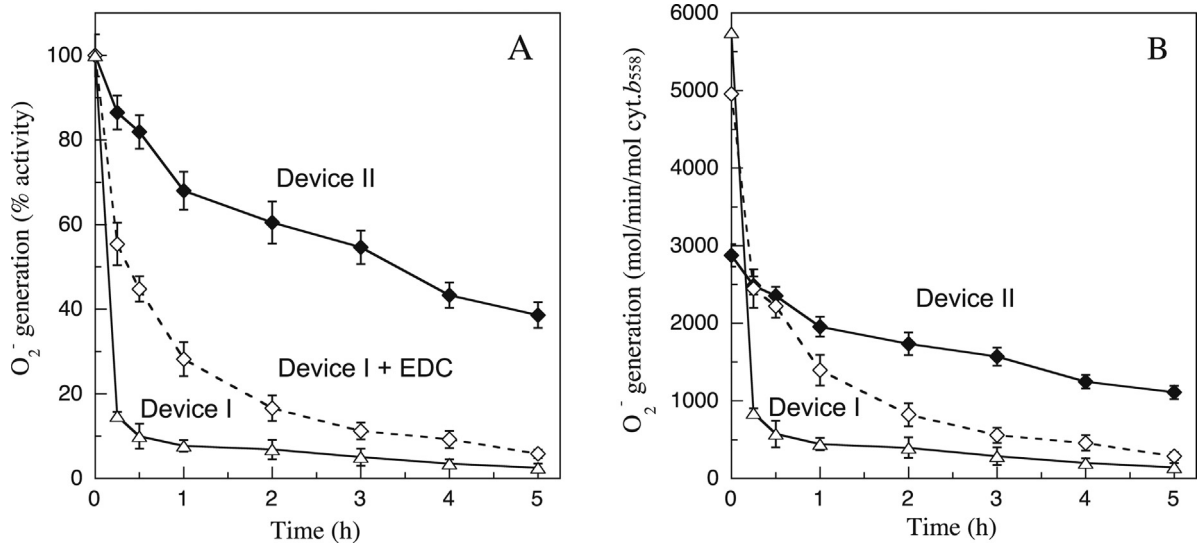

Fig. 2. Stability of O_2^- generating activities of Device I and Device II in MEM.
Each sample was diluted 23-fold with MEM (without phenol red) and incubated at 37 °C. An aliquot of the mixture (250 μl) containing 1 pmol of cyt.b_{558} was taken at a given time and assayed for O_2^- generation. (A) The percentages of the activities of Device I (triangle), Device I treated with EDC (open diamond), and Device II (closed diamond) at a given incubation time. (B) The actual O_2^--generating activities of Device I (triangle), Device I treated with EDC (open diamond), and Device II (closed diamond).

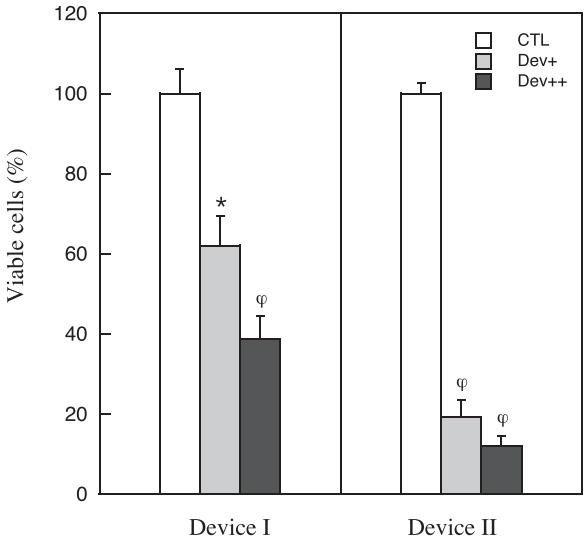

Fig. 3. Effect of Device I and Device II on viability of HEK293 cells. HEK293 cells (2×10^5) were cultured in the presence of NADPH and Device I or II including 0.8 pmol of cyt.b_{558} (Dev+) or 2 pmol of cyt.b_{558} (Dev++) at 37 °C in MEM (400 μl). Control cells were cultured in the absence of device and NADPH (CTL). After 24 h, the numbers of live cells counted are expressed as the means ± S.D. ($n = 4$). The result is a typical one of three independent experiments. *: $P < 0.005$ *vs* control; ϕ: $P < 0.001$ *vs* control.

Device II was added without NADPH, the percentage of surviving cells did not differ from control cells (Fig. 3).

3.4. Identification of the ROS causing the cell death induced by Device II

To clarify whether the ROS causing the cell death of HEK293 cells was O_2^- or H_2O_2, we added Device II with SOD to the system (Fig. 4). SOD addition did not decrease the cell death, but slightly enhanced it. On the other hand, when both SOD and catalase were added to the system, the cell viability was recovered

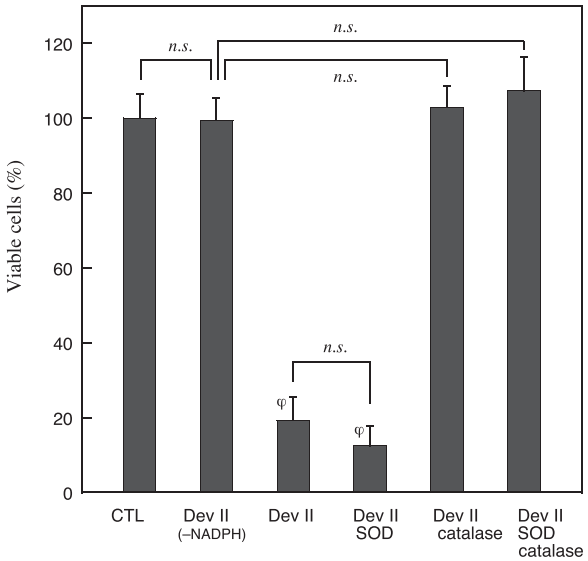

Fig. 4. Effect of SOD and catalase on Device II-induced cell death of HEK293 cells. HEK 293 cells (2×10^5) were incubated at 37 °C for 24 h with Device II and NADPH (Dev II), Device II alone (Dev II*), or buffer A (CTL) in 400 μl MEM. In some experiments the mixture was supplemented with SOD or SOD plus catalase. Other experimental conditions were as described under experimental sections. The live cell numbers are expressed as percentages of that with the control cells after 24 h-incubation (2.4×10^5). Data represent the means ± S.D. ($n = 4$). The data shown is a typical one of three independent experiments. *n.s.*: not significant; ϕ: $P < 0.001$ *vs* Device II (-NADPH).

to the level of untreated cells. The addition of catalase without the device did not affect the viability of the cells (93% of the control). These data indicated that H_2O_2, and not O_2^-, caused the cell death of HEK293 cells, as shown for Device I [22].

3.5. Detection of caspase-3 activation

To investigate whether the cell death induced by Device II proceeded through apoptosis, we examined caspase-3 activation by flow cytometric analyses using NV, a caspase-3 substrate that sheds fluorescence when cleaved. Fig. 5A shows typical flow cytometric diagrams after incubation for 24 h with or without the device. The mean populations of caspase-3-activated cells are summarized in Fig. 5B. The population of NV(+) (caspase-3-activated) cells increased from 7% to 44% after treatment with the device. Meanwhile, the population of PI(+) (membrane-integrity-impaired) cells increased from 4% to 38%. These results suggested that the cell death largely proceeded through apoptosis. The slightly higher percentage of NV(+) cells than PI(+) cells indicated that some cells were in an early apoptotic state at 24 h of treatment (Fig. 5B).

4. Discussion

H_2O_2 has often been used in oxidative stress studies on cultured cells. However, as mentioned above, the initially formed ROS *in vivo* is O_2^- in most cases. Although O_2^- is soon converted to H_2O_2 spontaneously or enzymatically, there may be a mixture of H_2O_2 and O_2^- outside of the cells. In addition, reactions between these two agents can lead to the formation of $OH^\bullet$ or $^1O_2$, which are not formed from H_2O_2 alone [11]. Therefore, addition of O_2^- would more closely mimic the oxidative stress induced by ROS in biological systems.

Xanthine oxidase has been used for this purpose, but the enzyme has several demerits for cell experiments: (i) poor solubility of substrates; (ii) possible conversion to xanthine dehydrogenase; and (iii) relatively high pH optimum [22]. More seriously, the enzyme produces urate as a product, which scavenges $OH^\bullet$ and $^1O_2$ [1].

We previously developed an O_2^--generating nanodevice consisting of Nox2 and its activating factors [22], which has advantages over xanthine oxidase in the above points. However, the device was subsequently found to be unstable in MEM. The present study showed that the stability was somewhat improved by EDC but was not enough to retain the activity for several hours. The stability was markedly improved by addition of sulfo-NHS, a coupling enhancer, which may protect against hydrolysis of the acyl intermediate [18]. The result was somewhat unexpected, because EDC by itself was previously found to be effective in the stabilization of Nox2 against thermal treatment [20,21] or dilution [15]. Therefore, it appears that the inactivation mechanism by MEM differs from that in the above conditions. Further studies will be required to clarify this point.

To examine how EDC/NHS treatment stabilized the oxidase, we performed western blotting analyses on the Device II mixture using antibodies against its components. Device II consisted of Nox2, $p22^{phox}$, RacQ61L, and $p67^{phox}$-$p47^{phox}$, similar to Device I [22]. Western blotting analysis of Device II with an anti-Rac antibody revealed new bands at 78 and 112 kD, while that with an anti-$p67^{phox}$ antibody showed three new bands at 78, 148, and 169 kD, suggesting that EDC produced cross-links among Rac (21 kD), $p67^{phox}$–$p47^{phox}$ (57 kD), and Nox2 (91 kD). However, the intensities of the newly appeared bands were much weaker than those of the original bands. In addition, western blotting with antibodies against Nox2 or $p22^{phox}$ showed no other bands than those for

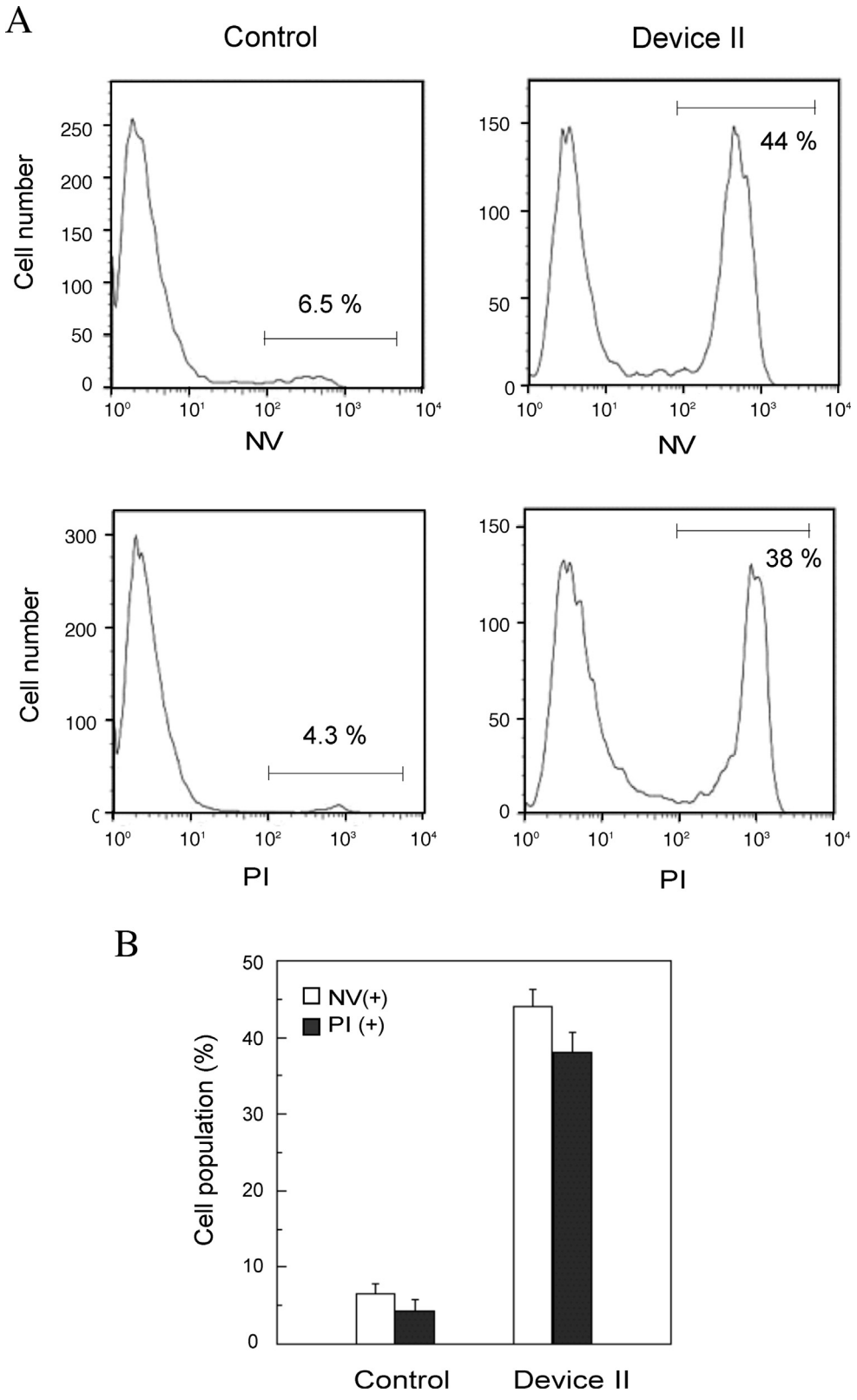

Fig. 5. Caspase-3 activation in HEK293 cells exposed to Device II.
HEK 293 cells (2×10^5) were incubated at 37 °C for 24 h with or without Device II (0.4 pmol cytochrome b_{558} equivalent) and NADPH. (A) Flow cytometric analysis. The cells were analyzed with a flow cytometer by staining with NV or PI. The results shown are representative of three independent experiments. (B) Summary of flow cytometry. The data are expressed as percentages of NV-positive cells (open bars) and PI-positive cells (closed bars). Values are the means ± S.D. of three determinations.

the original proteins at 91 and 22 kD, respectively. It is plausible that the haptens for these antibodies were deeply buried within the cross-linked complex and became inaccessible to the antibodies. Another possibility is that cross-linking might occur intramolecularly within some of the components. Thus, the mechanism of the complex stabilization by EDC/NHS is not clear at the present time, and further studies are required to clarify this issue.

Device II efficiently induced cell death of HEK293 cells. The extent was greater than that with the original device, Device I (Fig. 3). In addition, flow cytometric analyses showed that the Device II-induced cell death largely proceeded through apoptosis. Although H_2O_2-induced apoptosis has often been reported in mammalian cell lines [7,23], including HEK293 cells [23], there are few reports demonstrating that added O_2^- induces apoptosis except for our previous paper [22]. As mentioned above, the ROS generated *in vivo* is O_2^-. This is the case not only in host defense and inflammatory responses, but also in intracellular signaling. Specifically, for elevation of the intracellular H_2O_2 level, it is assumed that endogenous Nox enzyme releases O_2^- from the cells, which is converted to H_2O_2 in extracellular space, and then the resulting H_2O_2 penetrates the membrane through specific aquaporins [12]. Therefore, we believe that addition of O_2^- can mimic *in vivo* situations more closely.

We confirmed that the ROS inducing cell death in HEK293 cells was mainly H_2O_2. However, this does not necessarily mean that O_2^- has no specific role other than as a precursor of H_2O_2. It may oxidize proteins and lipids on the membrane and possibly change the membrane integrity. In addition, two groups demonstrated that chloride channels including ClC-3 may transport O_2^- through the plasma membrane [8] or endosomal membrane [17] of certain cell types. Therefore, the specific roles of O_2^- *in vivo* are interesting subjects that remain to be clarified.

In summary, we established a new version of our previous O_2^--generating nanodevice, designated Device II. The new version was stable in MEM and produced high levels of O_2^- continuously. Device II induced apoptosis in HEK293 cells through H_2O_2. We believe this new nanodevice will be useful for mimicking *in vivo* situations in which cells are exposed to high levels of O_2^- during host defense, inflammatory responses, or accidental formation.

Acknowledgments

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