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Original Article

The paradoxical effect of 1,4-naphthoquinone on the process of cell death induced by hydrogen peroxide in rat thymocytes

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ABSTRACT — 1,4-Naphthoquinone (NAPH) is found in diesel exhaust particles and it is an active metabolite of naphthalene, a fumigant insecticide. This compound is known to cause oxidative stress. Therefore, it is plausible to suggest that NAPH increases cell vulnerability to oxidative stress in an additive or synergistic manner. We tested this possibility using rat thymocytes with flow-cytometric techniques and appropriate fluorescent probes. NAPH attenuated the increase in cell lethality induced by hydrogen peroxide (H_2O_2). The combination of NAPH and H_2O_2 promoted the transition from normal cells to apoptotic living cells, but attenuated further transition to cell death. Thus, the process of cell death induced by H_2O_2 was not completed in the presence of NAPH. However, NAPH did not attenuate certain lethal cellular events such as decrease in the cellular content of non-protein thiols and increases in intracellular Ca^{2+} and Zn^{2+} levels, induced by H_2O_2. The inhibitory effect of NAPH on the increase in cell lethality induced by H_2O_2 was also observed when caspase activity was suppressed. In the present study, the mechanism underlying the NAPH-induced attenuation of cell death in cells affected by H_2O_2-generated oxidative stress was, however, not fully elucidated. Since both H_2O_2 and NAPH elevated intracellular Ca^{2+} and Zn^{2+} levels, and since Zn^{2+} is known to partly attenuate Ca^{2+}-dependent cell death, the intracellular interaction between Ca^{2+} and Zn^{2+} may complicate the process of cell death induced by oxidative stress.

Key words: 1,4-Naphthoquinone, Oxidative stress, Cell death, Hydrogen peroxide

INTRODUCTION

1,4-Naphthoquinone (NAPH) is an active metabolite of naphthalene, a fumigant insecticide, (Agency for Toxic Substances and Disease Registry, 2005; Cho *et al*., 2006) and it is also found in diesel exhaust particles (Cho *et al*., 2004). NAPH causes *in vitro* and *in vivo* toxicities (Wilson *et al*., 1996; Lamé *et al*., 2003; Ishihara *et al*., 2011) *via* the formation of reactive oxygen species (Kumar *et al*., 2009; Srinivas *et al*., 2011). In our previous study (Fukunaga *et al*., 2014), we showed that NAPH at sublethal concentrations increased the intracellular Zn^{2+} levels in rat thymocytes. This phenomenon is one of the common features of cells affected by oxidative stress because the conversion of thiols to disulfides by oxidative stress releases Zn^{2+} (Maret, 1994), resulting in an increase in intracellular Zn^{2+} levels (Kinazaki *et al*., 2011). An excessive increase in intracellular Zn^{2+} level has a critical role in the cytotoxicity of hydrogen peroxide (H_2O_2), one of reactive oxygen species, because the chelator of intracellular Zn^{2+} reduces the cytotoxicity of H_2O_2 (Matsui *et al*., 2010). Therefore, it is plausible to suggest that NAPH increases cell vulnerability to oxidative stress in an additive or synergistic manner. Here, we tested this hypothesis and showed that NAPH at sublethal concentrations attenuated the increase in H_2O_2-induced cell lethality and exhibited paradoxical actions on the process of cell death induced by H_2O_2.

MATERIALS AND METHODS

Reagents

NAPH was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Propidium iodide, annexin

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V-fluorescein isothiocyanate (Annexin V-FITC), 5-chloromethylfluorescein diacetate (5-CMF-DA), Fluo-3-*tetra*(acetoxymethyl) ester (Fluo-3-AM), FluoZin-3-AM, *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol (Oxonol), and benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) were purchased from Molecular Probes Inc. (Eugene, OR, USA). The pH buffer used in this study was 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Nacalai Tesque, Kyoto, Japan). Chelator of intracellular Zn^{2+}, *N,N,N′,N′-tetrakis*(2-pyridylmethyl)ethylenediamine (TPEN) was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). NAPH, oxonol, 5-CMF-DA, Fluo-3-AM, and FluoZin-3-AM were initially dissolved in dimethyl sulfoxide (DMSO). DMSO (0.3%) did not affect cell viability or the fluorescence measurements.

Animals and cell preparation

This study was approved by the Committee for Animal Experiments in the University of Tokushima, Tokushima, Japan (No. 05279 and No. 15124). The experimental animals were kept under standard rearing conditions.

Cell suspensions were prepared as previously reported (Chikahisa *et al*., 1996; Matsui *et al*., 2010). Briefly, thymus glands were dissected from 6- to 12-week-old Wistar rats and sliced at a thickness of about 1 mm. The slices were gently triturated in chilled Tyrode's solution (150 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 5 mM glucose; 5 mM HEPES, adjusted to pH 7.3-7.4 with 2.5 mM NaOH) to dissociate lymphocytes. Thereafter, the solution containing the cells was passed through a mesh (diameter: 56 μm) to prepare the cell suspension (approximately 5×10^5 cells/mL). The cells were incubated at 36-37°C for 1 hr before use. It has been shown that the cell suspension prepared under these conditions contains 0.2-0.23 μM zinc derived from the cell preparation (Sakanashi *et al*., 2009).

Fluorescence measurements of cellular parameters

Cell and membrane parameters were measured using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes (Chikahisa *et al*., 1996; Sakanashi *et al*., 2009; Matsui *et al*., 2010). The excitation wavelength for the fluorescent probes used in this study was 488 nm. The emissions were detected at 530 ± 20 nm for FITC, 5-CMF, Fluo-3, FluoZin-3, and Oxonol fluorescence and at 600 ± 20 nm for propidium fluorescence. Fluorescence was analyzed using the JASCO software (Version 3.06; JASCO). 5-CMF, Fluo-3, FluoZin-3, and Oxonol fluorescence were monitored from intact living cells that were not stained with propidium. The reagents used in the study were non-fluorescent, except for the fluorescent probes, under the experimental conditions.

To assess cell viability using propidium iodide, the dye was added to the cell suspension at a final concentration of 5 μM. Since propidium stains dead cells and/or cells with compromised membranes, the measurement of propidium fluorescence from cells assesses cell viability. The exposure of phosphatidylserine on the outer cell membrane surface, one of the events during the early stage of apoptosis, was detected using annexin V-FTIC (Koopman *et al*., 1994). The cells were incubated with annexin V-FITC (10 μL/mL) for 30 min and with propidium iodide (5 μM) for 2 min before the fluorescence measurements. Changes in cellular content of non-protein thiols, presumably glutathione, were monitored using 5-CMF-DA (Chikahisa *et al*., 1996). The cells were incubated with 1 μM 5-CMF-DA for 30 min before the fluorescence measurements. Changes in intracellular Ca^{2+} and Zn^{2+} levels were estimated by incubating the cells with 500 nM Fluo-3-AM (Kao *et al*., 1989) and FluoZin-3-AM (Gee *et al*., 2002), respectively, for 60 min before the fluorescence measurements. Alterations in the membrane potential of living cells were monitored with Oxonol (Wilson and Chused, 1985). Oxonol was added to the cell suspension at a final concentration of 500 nM. Shifts toward increased and decreased fluorescence intensities corresponded with depolarization and hyperpolarization of the membrane potential, respectively.

Experimental protocol

NAPH (0.1-1 mM of 2 μL DMSO solution) was added to cell suspensions (2 mL per test tube) that were incubated at 36-37°C. The incubation was prolonged with the agent for 1-3 hr. Cell suspension (a volume of 100 μL) was analyzed using flow cytometry to assess the NAPH-induced changes in cellular and membrane parameters.

Cell death was induced with 1 mM H_2O_2. The incubation time with H_2O_2 to induce cell death varied from preparation to preparation. Therefore, the effect of NAPH on cell lethality was determined 3 hr after the start of the simultaneous application of H_2O_2 to the cells under the condition that the cell lethality should exceed 20% at 2 hr after the application of H_2O_2.

Statistical analysis

Statistical analysis was performed using Tukey's multivariate analysis. A P value of < 0.05 was considered significant. Values (columns and bars in figures) are

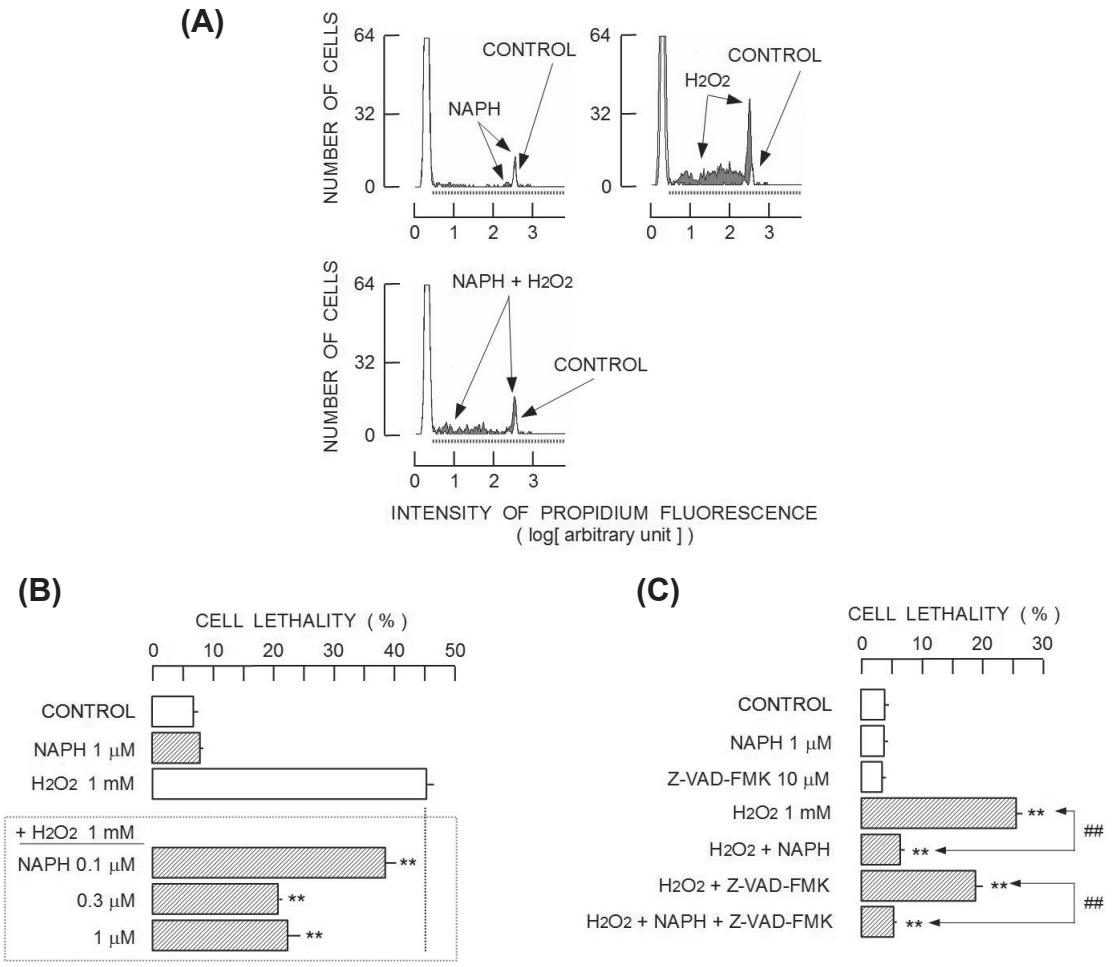

Fig. 1. The change in cell lethality by hydrogen peroxide (H_2O_2) in the absence or presence of 1,4-naphthoquinone (NAPH). (A) The changes in the histograms of the propidium fluorescence in the presence of 1 μM NAPH, 1 mM H_2O_2, or both. The effects were examined 3 hr after the start of drug application. Each histogram was constructed with the data obtained from 2500 cells. (B) The changes in cell lethality induced by NAPH, H_2O_2, or their combination. Asterisks (**) indicate a significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 and the group of cells treated with H_2O_2 and NAPH. (C) The change in cell lethality induced by NAPH, H_2O_2, and their combination in the absence and presence of benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK). Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the compound-treated groups. The symbols (##) indicate a significant difference ($P < 0.01$) between the paired groups.

expressed as the mean ± standard deviation of four samples. The experiments were repeated three times unless mentioned.

RESULTS

The effect of NAPH on the H_2O_2-induced increase in cell lethality

In our previous study, NAPH significantly increased cell lethality at a concentration ≥ 3 μM (Fukunaga *et al*., 2014). The experiments in the current study were carried out with sublethal concentrations (0.1-1 μM) of NAPH. In contrast to that with 1 mM H_2O_2, incubation with 1 μM NAPH for 3 hr did not increase the population of cells stained with propidium iodide (Fig. 1A). Furthermore, the increase in the population of cells exhibiting propidium fluorescence, dead cells, after the simultaneous application of NAPH and H_2O_2 was smaller than that with H_2O_2 alone (Fig. 1A). The results of the simultaneous application of H_2O_2 and NAPH (0.1-1 μM) are sum-

marized in Fig. 1B and show that NAPH seems to attenuate the H_2O_2-induced increase in the population of dead cells.

Z-VAD-FMK, a pan-caspase inhibitor, can reveal the existence of alternative backup cell death programs for apoptosis (Vandenabeele *et al*., 2006). Therefore, the effects of NAPH, H_2O_2, and their combination on cell lethality were tested in the presence of 10 μM Z-VAD-FMK. Although Z-VAD-FMK reduced the increase in cell lethality caused by incubation with 1 mM H_2O_2 for 3 hr, 1 μM NAPH similarly attenuated the H_2O_2-induced increase in cell lethality in the presence of Z-VAD-FMK (Fig. 1C).

The effect of NAPH on the change in cell population by H_2O_2

The results described above may indicate that NAPH, a pro-oxidant, can reduce the cytotoxicity of H_2O_2. Therefore, it is interesting to examine the effect of NAPH on the process of H_2O_2-induced cell death. Incubation with NAPH (1 μM) for 2 hr decreased the population of intact living cells (area N of Fig. 2) and increased that of annexin V-positive living cells (area A of Fig. 2). Hydrogen peroxide (1 mM) induced a profound transition of the cells from area N to A, indicative of a shift toward apoptosis. The simultaneous application of NAPH and H_2O_2 further accelerated the transition from intact living cells to cells that were positive for annexin V (Fig. 2). However, an increase in the population of cells exhibiting propidium fluorescence was not observed (Fig. 2).

The effects of NAPH on the cellular thiol content and the intracellular Ca^{2+} and Zn^{2+} levels

Hydrogen peroxide decreases the cellular content of non-protein thiols (mainly glutathione) and increases intracellular Ca^{2+} and Zn^{2+} levels (Okazaki *et al*., 1996; Chikahisa *et al*., 1996; Matsui *et al*., 2010). These phenomena are linked to cell death. Therefore, the effects of 1 μM NAPH, 1 mM H_2O_2, and their combination on 5-CMF, Fluo-3, and FluoZin-3 fluorescence were examined 1 hr after the start of their application to the cells. As shown in Fig. 3A, both agents diminished the 5-CMF fluorescence, which was almost completely diminished after their combined application. Incubation with 1 μM

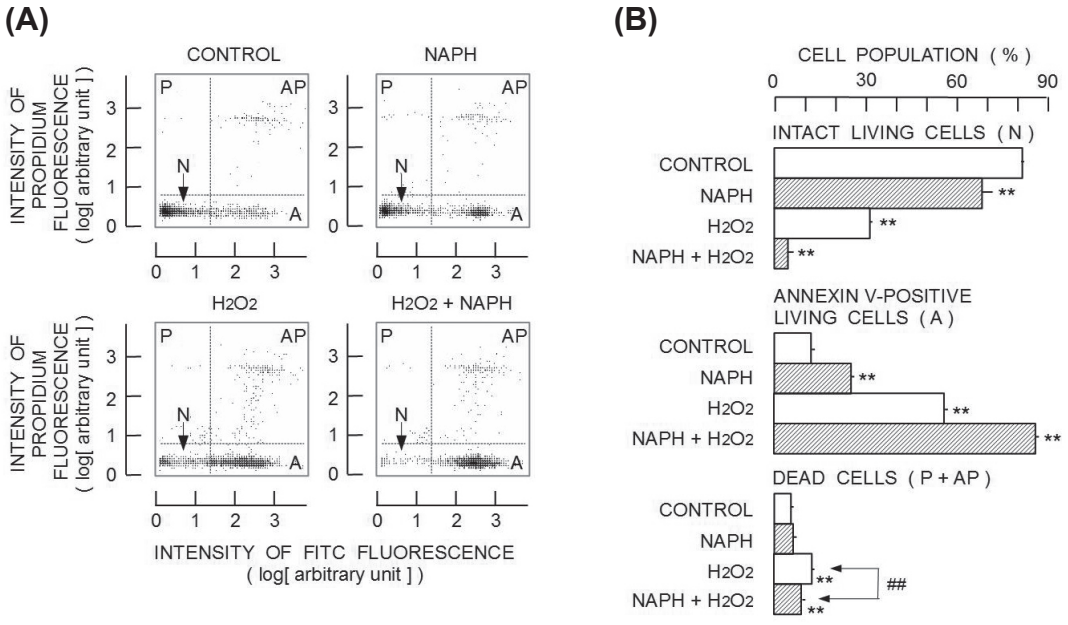

Fig. 2. The changes in the cell populations (intact living cells, annexin V-positive living cells, and dead cells) classified by propidium iodide and annexin V-FITC fluorescence. (A) Fluorescence cytograms (propidium fluorescence versus FITC fluorescence) were obtained from cells incubated with 1 μM NAPH, 1 mM H_2O_2, or their combination. Each cytogram was obtained from the data of 2000 cells. The areas N, A, P, and AP show the populations of intact living cells, annexin V-positive living cells, dead cells, and annexin V-positive dead cells, respectively. The effect was examined 2 hr after the start of the application compound(s). (B) The percentage change in cell population after a 2 hr incubation with NAPH, H_2O_2, and both. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the compound-treated groups. The symbols (##) indicate a significant difference ($P < 0.01$) between the arrowed groups of dead cells.

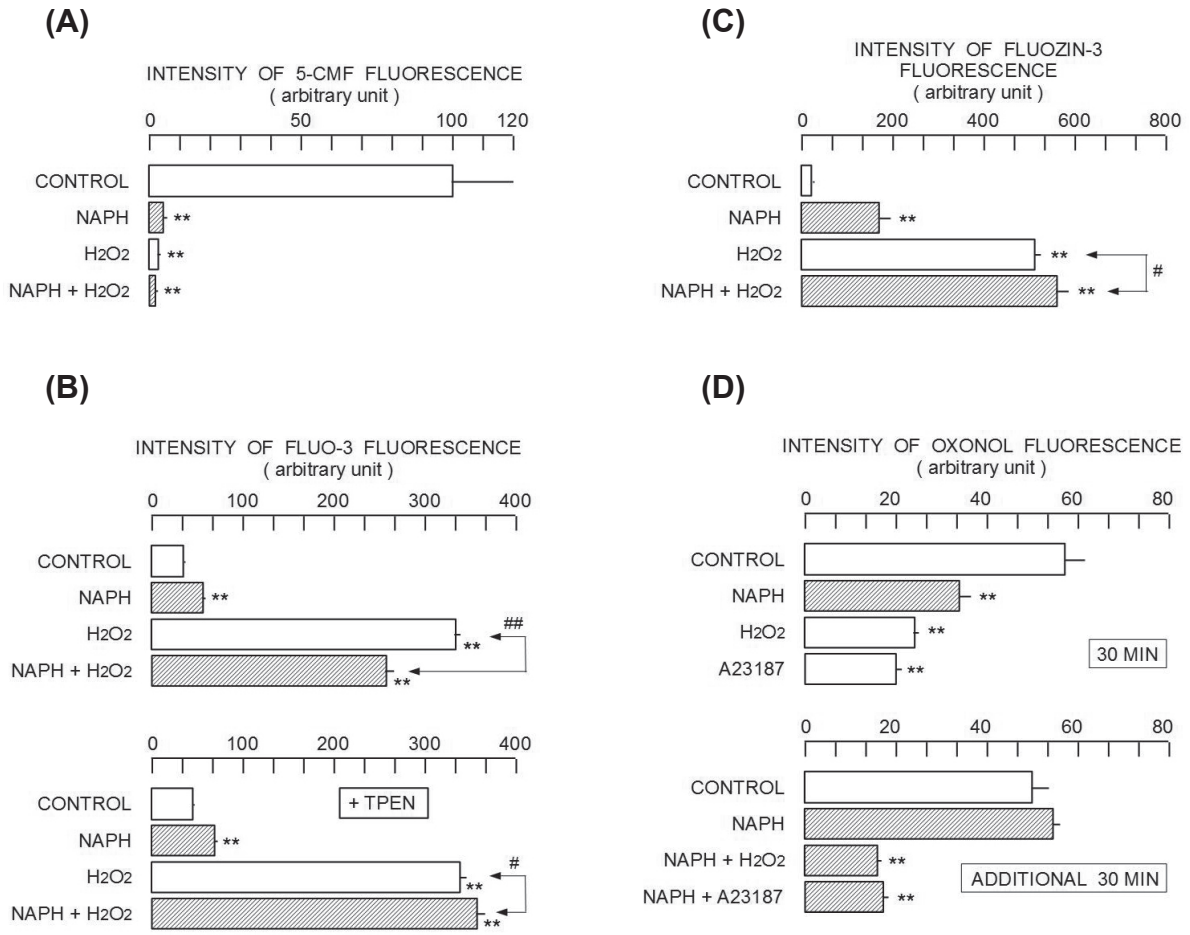

Fig. 3. The changes in 5-CMF, Fluo-3, and FluoZin-3 fluorescence intensity due to NAPH, H_2O_2, and their combination. The effect was examined 1 hr after the start of the application of the compound(s). (A) The effects on 5-CMF fluorescence. (B) The effects on Fluo-3 fluorescence in the absence (upper panel) and presence (lower panel) of TPEN. (C) The effects on FluoZin-3 fluorescence. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the compound-treated groups. The symbols (##) indicate a significant difference ($P < 0.01$) between the paired groups. (D) The effects of NAPH, H_2O_2, their combination, and A23187 on oxonol fluorescence. Upper panel: the effects were determined 30 min after the start of the application of the respective compound(s). Subsequently, 1 μM NAPH was applied to the cells incubated with respectively 1 mM H_2O_2 and 300 nM A23187. Lower panel: the effects were examined after incubation for an additional 30 min. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the compound-treated groups.

NAPH for 1 hr slightly increased the Fluo-3 fluorescence intensity while it was significantly increased after incubation with 1 mM H_2O_2 (Fig. 3B). The simultaneous incubation with NAPH and H_2O_2 increased the fluorescence to a lesser extent compared to that with H_2O_2 alone. Both NAPH and H_2O_2 are known to increase intracellular Zn^{2+} levels in rat thymocytes (Matsui *et al*., 2010; Fukunaga *et al*., 2014). If the elevation of the intracellular Zn^{2+} level would disturb the binding of Ca^{2+} to Fluo-3 because of the higher affinity of Zn^{2+} to Fluo-3, it would attenuate the augmentation of the Fluo-3 fluorescence. Cells incubated with the combination of NAPH and H_2O_2 for 1 hr showed a further increase in the FluoZin-3 fluorescence intensity (Fig. 3C). Therefore, the effect of NAPH on Fluo-3 fluorescence was tested in the presence of TPEN (10 μM), a chelator of intracellular Zn^{2+}. As shown in Fig. 3B, the Fluo-3 fluorescence intensity monitored from the cells incubated with both NAPH and H_2O_2 was slightly, but

significantly, higher than that of the cells incubated with H_2O_2 alone. Thus, these results seem to be influenced by the elevation of intracellular Zn^{2+} levels (Fig. 3C). However, they do not explain the mechanism underlying the suppression of H_2O_2-induced cell death by NAPH.

The effects of NAPH on the cell membrane potential

Charybdotoxin and clotrimazole, Ca^{2+}-activated K^+ channel blockers, protect cells from cell death caused by an excessive increase in intracellular Ca^{2+} levels (Horimoto *et al*., 2006). If NAPH blocks Ca^{2+}-dependent hyperpolarization, it would protect against H_2O_2-induced cell death. NAPH initially decreased the intensity of Oxonol fluorescence (hyperpolarization) and the intensity returned to the control level (Fig. 3D). Hydrogen peroxide (1 mM) induced the hyperpolarization in the absence and presence of 1 μM NAPH (Fig. 3D). This phenomenon was also observed in the case of 300 nM A23187, a calcium ionophore (Fig. 3D). Thus, these findings suggest that NAPH does not inhibit the Ca^{2+}-dependent hyperpolarization.

DISCUSSION

The paradoxical effect of NAPH

NAPH attenuated the increase in cell lethality induced by H_2O_2 (Fig. 1). The combination of NAPH and H_2O_2 promoted the transition from normal cells to annexin V-positive living cells, but attenuated further transition to dead cells (Fig. 2). Thus, the H_2O_2-induced process of cell death was not completed in the presence of NAPH in some cells. It is unlikely that NAPH attenuated the lethal cellular events triggered by H_2O_2 because of the following phenomena. The combination of H_2O_2 with NAPH changed the cellular content of non-protein thiols (Fig. 3A), intracellular Ca^{2+} (Fig. 3B) and Zn^{2+} levels (Fig. 3C) more than those observed for H_2O_2 alone. Previous work has shown that these changes were related to H_2O_2-induced cell death in rat thymocytes (Okazaki *et al*., 1996; Matsui *et al*., 2010). Furthermore, NAPH did not affect hyperpolarization induced by H_2O_2 and A23187 (Fig. 3D). Thus, NAPH does not inhibit Ca^{2+}-dependent K^+ channels. Charybdotoxin, a specific inhibitor of Ca^{2+}-dependent K^+ channels, greatly attenuates Ca^{2+}-induced cell death in rat thymocytes (Horimoto *et al*., 2006). Therefore, we can rule out the possibility that NAPH attenuated H_2O_2-induced lethal cellular events resulting in decreased cell lethality.

Chemicals can trigger several cell death processes (Galluzzi *et al*., 2014). Furthermore, the suppression of one cell death pathway seems to activate another, alternative pathway. For example, although the addition of a pan-caspase inhibitor blocks apoptotic cell death, it sensitizes cells to induce necrotic cell death and/or autophagic cell death (Vandenabeele *et al*., 2006). As shown in Fig. 1C, the increase in H_2O_2-induced cell death was slightly, but significantly, attenuated in the presence of Z-VAD-FMK. The inhibitory effect of NAPH on the H_2O_2-induced increase in cell lethality was similarly observed even in the presence of Z-VAD-FMK. It is, therefore, suggested that NAPH attenuated the caspase-independent pathway of H_2O_2-induced cell death. The cell death in the presence of Z-VAD-FMK seems to be necrotic or autophagic. In the present study, the mechanism underlying the NAPH-induced attenuation of cell death in cells treated with H_2O_2 has not been elucidated. However, we speculate that NAPH inhibits (or delays) late stage of cell death during necrosis or autophagy.

Toxicological implications

It is not surprising that the combination of two cytotoxic substances induces less potent cytotoxicity in rat thymocytes. The increase in cell lethality by the combination of tributyltin, a highly toxic biocide, and H_2O_2 was less than that by H_2O_2 alone (Sakai *et al*., 2001). A23187, a calcium ionophore, partly attenuated the increase in cell lethality by H_2O_2 (Oyama *et al*., 2003). Thus, tributyltin and A23187 seem to block or delay the process of cell death induced by H_2O_2. The combination of toxic compounds (or multiple pollution) does not always exhibit an additional or synergic cytotoxic action. Hydrogen peroxide, tributyltin, A23187, and NAPH increase intracellular Ca^{2+} and Zn^{2+} levels (Chikahisa and Oyama, 1992; Okazaki *et al*., 1996; Oyama *et al*., 2009; Fukunaga *et al*., 2014). Zn^{2+} partly attenuates Ca^{2+}-dependent cell death (Sakanashi *et al*., 2009). Therefore, the intracellular interaction between Ca^{2+} and Zn^{2+} may complicate the process of cell death induced by chemical compounds that elevate intracellular Ca^{2+} and Zn^{2+} levels.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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