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 (H2O2). The combination of NAPH and H2O2 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 H2O2 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 Ca2+ and Zn2+ levels, induced by H2O2. The inhibitory effect of NAPH on the increase in cell lethality induced by H2O2 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 H2O2-generated oxidative stress was, however, not fully elucidated. Since both H2O2 and NAPH elevated intracellular Ca2+ and Zn2+ levels, and since Zn2+ is known to partly attenuate Ca2+-dependent cell death, the intracellular interaction between Ca2+ and Zn2+ 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 Zn2+ 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 Zn2+ (Maret, 1994), resulting in an increase in intracellular Zn2+ levels (Kinazaki et al., 2011). An excessive increase in intracellular Zn2+ level has a critical role in the cytotoxicity of hydrogen peroxide (H2O2), one of reactive oxygen species, because the chelator of intracellular Zn2+ reduces the cytotoxicity of H2O2 (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 H2O2-induced cell lethality and exhibited paradoxical actions on the process of cell death induced by H2O2.

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 benzoyloxy carbonyl-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 6-12-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 $5 \times 10^5$ 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-FITC (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$_2$O$_2$. The incubation time with H$_2$O$_2$ 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$_2$O$_2$ to the cells under the condition that the cell lethality should exceed 20% at 2 hr after the application of H$_2$O$_2$.

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
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$_2$O$_2$-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$_2$O$_2$, 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$_2$O$_2$ was smaller than that with H$_2$O$_2$ alone (Fig. 1A). The results of the simultaneous application of H$_2$O$_2$ and NAPH (0.1-1 μM) are sum-
marized in Fig. 1B and show that NAPH seems to attenuate the H$_2$O$_2$-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$_2$O$_2$, 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$_2$O$_2$ for 3 hr, 1 μM NAPH similarly attenuated the H$_2$O$_2$-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$_2$O$_2$

The results described above may indicate that NAPH, a pro-oxidant, can reduce the cytotoxicity of H$_2$O$_2$. Therefore, it is interesting to examine the effect of NAPH on the process of H$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$, 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](image-url)
NAPH for 1 hr slightly increased the Fluo-3 fluorescence intensity while it was significantly increased after incubation with 1 mM H₂O₂ (Fig. 3B). The simultaneous incubation with NAPH and H₂O₂ increased the fluorescence to a lesser extent compared to that with H₂O₂ alone. Both NAPH and H₂O₂ are known to increase intracellular Zn²⁺ levels in rat thymocytes (Matsui et al., 2010; Fukunaga et al., 2014). If the elevation of the intracellular Zn²⁺ level would disturb the binding of Ca²⁺ to Fluo-3 because of the higher affinity of Zn²⁺ to Fluo-3, it would attenuate the augmentation of the Fluo-3 fluorescence. Cells incubated with the combination of NAPH and H₂O₂ 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 Zn²⁺. As shown in Fig. 3B, the Fluo-3 fluorescence intensity monitored from the cells incubated with respectively 1 mM H₂O₂ 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.
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$_2$O$_2$-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$_2$O$_2$ (Fig. 1). The combination of NAPH and H$_2$O$_2$ promoted the transition from normal cells to annexin V-positive living cells, but attenuated further transition to dead cells (Fig. 2). Thus, the H$_2$O$_2$-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$_2$O$_2$ because of the following phenomena. The combination of H$_2$O$_2$ 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$_2$O$_2$ alone. Previous work has shown that these changes were related to H$_2$O$_2$-induced cell death in rat thymocytes (Okazaki et al., 1996; Matsui et al., 2010). Furthermore, NAPH did not affect hyperpolarization induced by H$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$-induced cell death was slightly, but significantly, attenuated in the presence of Z-VAD-FMK. The inhibitory effect of NAPH on the H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ was less than that by H$_2$O$_2$ alone (Sakai et al., 2001). A23187, a calcium ionophore, partly attenuated the increase in cell lethality by H$_2$O$_2$ (Oyama et al., 2003). Thus, tributyltin and A23187 seem to block or delay the process of cell death induced by H$_2$O$_2$. 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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