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Research Article

Hydrogen peroxide and hydroxyl radical involvement in the activation of caspase-3 in chemically induced apoptosis of HL-60 cells

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Abstract. Apoptosis of HL-60 cells induced by actinomycin D, H7, or daunorubicin was shown to involve the activation of caspase-3-like protease, 2 h after the addition of these drugs, based on microassay of enzyme activity by high-performance liquid chromatography. Catalase and a spin trap, N-*t*-butyl-α-phenylnitrone, which effectively inhibited the apoptosis induced by these drugs, also inhibited the activation of caspase-3-like protease. These results suggest that hydrogen peroxide and the hydroxyl radical are common mediators of caspase-3 activation caused by these chemicals, with apparently different functional mechanisms. Based on mitochondrial activity determined by oxygen consumption, complexes I, II, and IV were inhibited by actinomycin D. H7 inhibited complexes I and IV, 1 and 1.5 h respectively, after the addition of the drug to HL-60 cells. Daunorubicin inhibited complex IV, 1.5 h after the addition of the drug to HL-60 cells. Inhibition of complex IV by actinomycin D, H7, and daunorubicin were almost fully restored by the addition of cytochrome c. The release to the cytosol of cytochrome c by these drugs was also demonstrated by Western blot analysis. Addition of catalase inhibited the depression of complex IV activity induced by actinomycin D and H7. These observations indicate a direct relationship between hydrogen peroxide and the release of cytochrome c during apoptosis caused by actinomycin D, H7, and daunorubicin.

Key words. Apoptosis; caspase-3; hydroxyl radical; HL-60; actinomycin D; hydrogen peroxide.

Since the first appearance of the term 'apoptosis' [1], great advances have been achieved in understanding the signalling systems of apoptosis [2], and a large body of evidence has accumulated to suggest that reactive oxygen species (ROS) play a key role as common mediators of apoptosis [3–5]. However, many problems remain to be clarified, for example, identifying the kind of inducers that use ROS as intracellular messengers of apoptosis, where, when, and how they are generated, and the mechanism by which ROS cause apoptosis. ROS have been suggested [6–7] to cause a mitochondrial permeability transition resulting in the release of caspase activators such as apoptosis-inducing factor [8] and cytochrome c [9]. However, Simizu et al. [10] recently reported that caspase-3 mediated the production of hydrogen peroxide in the apoptosis of human lung carcinoma Ms-1 cells induced by tyrosine kinase inhibitor and anticancer drugs [11]. The relationship between ROS and the activation of caspase is not well elucidated.

Recently, we reported [12] that apoptosis of HL-60 cells caused by actinomycin D, H7, or daunorubicin was significantly inhibited by catalase and a spin trap, N-*t*-butyl-α-

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phenylnitrone (PBN). These results suggest that hydrogen peroxide and the hydroxyl radical are common mediators of the apoptosis caused by these chemicals with apparently different functional mechanisms. In this report, we examined whether apoptosis caused by these drugs involves the activation of caspase-3 [13], and the involvement of ROS in the activation of the protease.

Materials and methods

RPMI 1640 medium (No. 22400), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from GIBCO BRL, Life Science Technologies. Actinomycin D, H7, daunorubicin, and the spin trap PBN were supplied by Sigma. Acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO) and acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA) were purchased from the Peptide Institute. All other reagents were purchased from Nacalai Tesque. Monoclonal anti-human cytochrome c antibody (clone 7H8.2C12) was purchased from Genzyme/Techne and goat anti-mouse IgG-HRP conjugate was from Santa Cruz Biotechnology.

Cell culture conditions

The human promyelocytic HL-60 leukemic cells were purchased from the Japan Health Sciences Foundation. Cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, penicillin (50 U/ml) and streptomycin (50 µg/ml) as described elsewhere [12]. The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere

Drug-induced apoptosis

Cells were pelleted by centrifugation at 600 × g for 2 min and resuspended at 1.0×10^6 cells/ml in RPMI 1640 medium without FBS. After the addition of actinomycin D dissolved in a mixture of ethylene glycol and ethanol (9:1) to a final concentration of 10 µg/ml [12], an aqueous solution of H7 (50 µM) [12], or daunorubicin (5 µM) [12], the cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 2, 4, and 6 h.

Microassay of caspase-3-like protease activity

Zero, 2, 4, and 6 h after the addition of an inducer, a 2-ml aliquot (2.0×10^6 cells) was taken and mixed with 2 ml of 100 mM Tris-HCl buffer at pH 7.5 containing 0.4% Triton X-100. After keeping on an ice bath for 30 min, the mixture was centrifuged at 15,000 × g for 5 min. To 1 ml of the supernatant were added 790 µl of a solution at pH 7.5 containing 100 mM of Tris-HCl, 2 mM of EDTA, 20 mM of EGTA, 200 µl of 10 mM dithiothreitol, and 10 µl of 10 mM DEVD-MCA solution. The resulting mixture was incubated at 37 °C. After 0 and 30 min, a 190-µl aliquot was taken, and the reaction was terminated by the addition of 10 µl of 5% $HClO_4$. After centrifugation, the supernatant (10 µl) was directly applied for high-performance liquid chromatography (HPLC) analysis to determine the release 7-amino-4-methylcoumarin (AMC). A reversed-phase column (µ-Bondasphere 5-µm C_{18}-100A, 3.9 × 150 mm; Waters) was eluted with a 1:4 mixture of acetonitrile and water containing 0.1% H_3PO_4 at a flow rate of 1.0 ml/min. Detection was made with a fluorescence detector (type RF-10AXL; Shimadzu) using excitation at 380 nm and emission at 460 nm. The caspase-3 reaction of the cells was confirmed as being linear at least for 1 h in that condition. Calibration was made by HPLC analysis using AMC solutions of a series of concentrations. The concentration range of AMC was chosen to give a linear relationship between the peak area and the AMC concentration. One unit (U) of enzyme activity was defined as 1 pmol AMC liberated/10^4 cells per minute.

Determination of apoptotic rate

Apoptotic cells were counted using a Nikon TMD300 fluorescence microscope after fixation and staining with Hoechst 33258 as described previously [12].

Inhibition experiments

Catalase (175 U/ml) was dissolved in RPMI 1640 medium, and cells were suspended in the resulting solution. The apoptosis inducer was added to the suspension and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 6 h. After the addition of inducers, a cell bottle was swirled gently for 1 min every 10 min during the first h and every 30 min thereafter [12].

PBN was dissolved in ethanol and added to the cell suspension at a final concentration of 20 mM. The final concentration of ethanol was 0.25%. The addition of PBN was followed by a 30-min preincubation at 37 °C for 30 min in a humidified atmosphere of 5% CO_2, and then inducers were added.

Determination of mitochondrial activities

Oxygen consumption was measured using a Clark-type oxygen electrode and a Rank Brothers polarograph with a thermojacketed chamber at 37 °C under constant stirring. The determination was essentially made as described previously [14]. Apoptosis was induced as described above. At the times indicated, a 30-ml aliquot was withdrawn, centrifuged, and washed twice with respiration buffer (0.25 M sucrose, 0.1% bovine serum albumin, 10 mM $MgCl_2$, 10 mM K^+Hepes, 5 mM KH_2PO_4, pH 7.2). Cells were suspended in 2 ml of the respiration buffer at a final concentration of 1.5×10^7 cells/ml and injected into a chamber. Oxygen consumption was measured with the sequential addition of substrates and inhibitors in the following order and final concentrations: ADP-K, 1 mM; malate, 50 mM; digitonin, 0.005%;

rotenone (an inhibitor of complex I), 100 nM; succinate, 5 mM; antimycin A (an inhibitor of complex III), 100 nM; tetramethyl-p-phenylenediamine (TMPD), 0.4 mM; ascorbate (ASC), 1 mM; and cytochrome c, 10 µM. The oxygen concentration was calibrated with air-saturated buffer assuming 212 µM. Four to five independent apoptotic processes were made to obtain the activity of each complex at each time.

Determination of cytochrome c in cytosol and mitochondria

After 1.5 h treatment of HL-60 cells with no inducer (control), actinomycin D, H7, or daunorubicin, the cells were harvested. Western blot analysis of cytochrome c in cytosol and mitochondria was performed essentially based on the method of Kamada et al. [15] except that digitonin at 0.1 mg/ml was used to separate the heavy membranes (containing mitochondria and nuclei) and the cytosolic fraction containing microsomes. Aliquots of the mitochondrial and cytosolic fractions derived from 4×10^6 cells were applied to SDS-PAGE electrophoresis. Detection was made with ECL Western blotting detection reagent purchased from Amersham Pharmacia Biotech.

Data are expressed as means ± SD for at least three independent runs and analyzed by ANOVA using StatView software (Abacus Concepts). Differences between group means were analyzed using Bonferroni/Dunn (Dunn's procedure as a multiple-comparison procedure) generated by this program. Differences are considered significant at $P < 0.05$.

Results and discussion

Apoptosis of HL-60 cells by actinomycin D

Actinomycin D-induced apoptosis of HL-60 cells is well documented [16–19]. The activity of caspase-3-like protease increased significantly ($P < 0.01$) from a barely detectable level (0.073 ± 0.075 U) at the start to 1.43 ± 0.38 U, 2 h; after the addition of actinomycin D, and reached a maximum level after 4 h (2.37 ± 0.32 U) (fig. 1a). After 6 h, enzyme activity was 1.87 ± 0.41 U. These results demonstrate that apoptosis caused by actinomycin D involves the activation of caspase-3. All caspase-3 activities determined in the present study were completely inhibited by 50 µM DEVD-CHO, a specific inhibitor of the enzyme [20]. Thus, the proteolytic activity observed in this study arose from caspase-3-like protease itself and not from other proteases.

Addition of catalase significantly inhibited the activation of caspase-3-like protease by actinomycin D after 4 h (1.36 ± 0.23 U) and 6 h (0.99 ± 0.17 U) (fig. 1a). This result indicates that hydrogen peroxide, a mediator of apoptosis of HL-60 cells induced by actinomycin D [12], is also involved in the activation of caspase-3, because catalase is a specific enzyme for decomposing hydrogen peroxide.

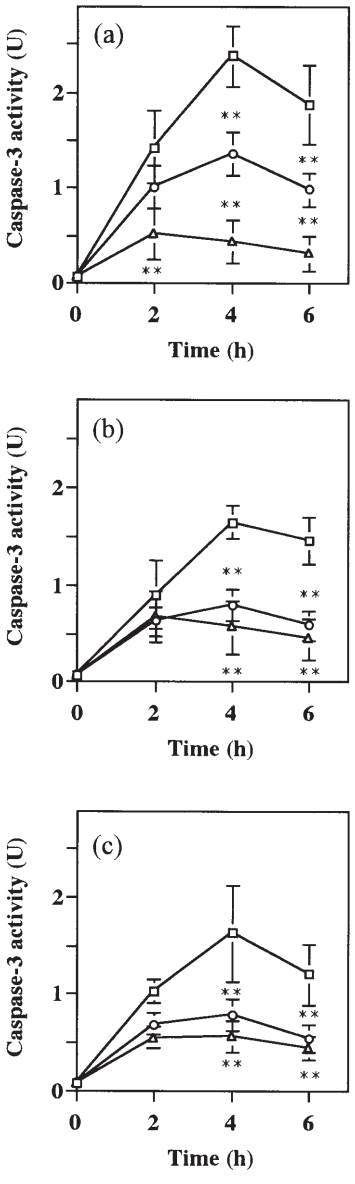

Figure 1. Activation of caspase-3-like protease in HL-60 cells by the inducers actinomycin D (*a*), H7 (*b*), and daunorubicin (*c*), and its inhibition by catalase and PBN 0, 2, 4, and 6 h after the addition of inducer (□), inducer and catalase (○), or inducer and PBN (△). Values are means ± SD, and asterisks indicate a significant difference from the corresponding control group (ANOVA Bonferroni/Dunn procedure, $*P < 0.05$ and $**P < 0.01$).

A spin trap, PBN at 20 mM, significantly inhibited the activation of caspase-3 2 h (0.52 ± 0.27 U), 4 h (0.44 ± 0.22 U), and 6 h (0.31 ± 0.18 U) after the addition of actinomycin D (fig. 1a). These results suggest that hydroxyl radicals are involved in the process leading to caspase-3 activation since hydrogen peroxide is suggested to be a mediator as described above, and readily generates hydroxyl radicals by a Fenton-type reaction.

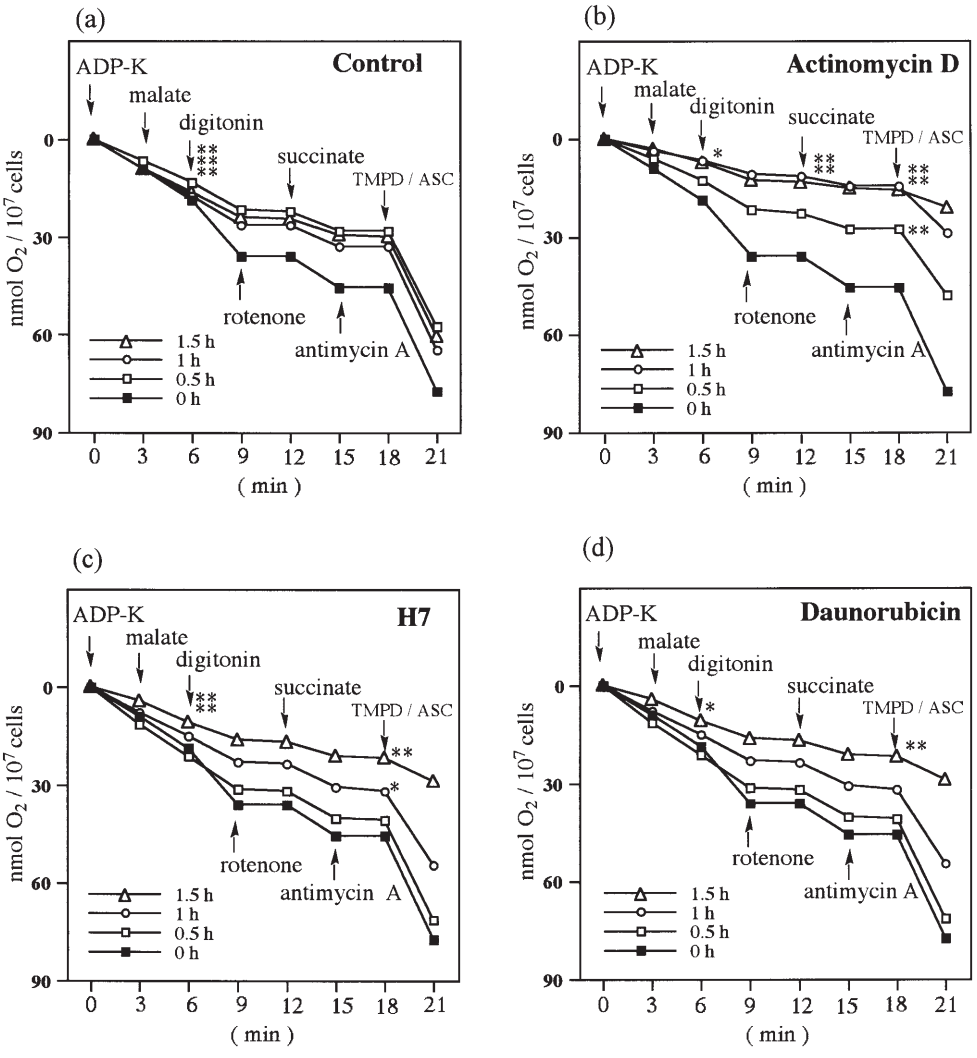

Figure 2. Mitochondrial activity of HL-60 cells incubated without the addition of apoptotic inducers. (*a*), and incubated with actinomycin D (*b*), H7 (*c*), and daunorubicin (*d*). Mitochondrial activity was assayed at the indicated times as described in the text. Values are means (SDs are smaller than the symbols), and asterisks indicate a significant difference from the activity of the control run at each time point (ANOVA Bonferroni/Dunn procedure, *P < 0.05 and **P < 0.01).

The apoptotic rate of HL-60 cells induced by actinomycin D after 6 h was 80.0 ± 3.1%, which was significantly reduced to 42.2 ± 6.8% and 31.0 ± 5.4% by catalase and PBN, respectively. These results were consistent with our previous study [12].

Mitochondrial activity was assayed based on oxygen consumption. Results for the control run without addition of inducers are shown in figure 2a. The activity of complex I decreased during incubation, but that of complex II and IV showed no change. To evaluate the effect of actinomycin D, a comparison was made for each time point with the control run. One hour after the addition of actinomycin D to HL-60 cells, the activities of complex I and II were significantly depressed (fig. 2b). After 1.5 h, complex II activity was still significantly suppressed (fig. 2b). Actinomycin D caused a marked decrease in the activity of complex IV as early as 0.5 h after the addition. After 1.5 h, the activity of complex IV was depressed to 29 ± 7% of the control value but was significantly restored to 88 ± 14% by 10 μM cytochrome c (fig. 3a). The recovered activity was not significantly different from the control value, i.e., the activity of complex IV was almost completely restored. This result clearly shows that the inactivation of complex IV was caused by the release of cytochrome c, and cytochrome oxidase seems intact 1.5 h after the addition of actinomycin D. The release of cytochrome c from mitochondria to the cytosol 1.5 h after the addition of actinomycin D was demonstrated by Western blot analysis (fig. 4). That cytochrome c release from mitochondria leads to the activation of caspase-3 is well documented [9]. Accordingly, activation of caspase-3 started approximately 2 h after the release of cytochrome c (fig. 1a).

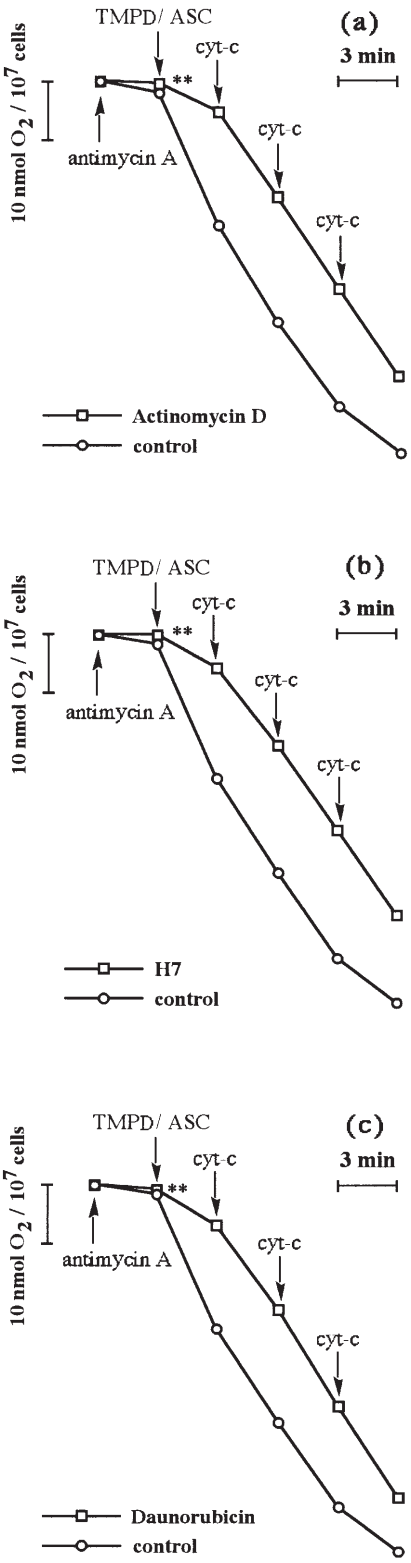

Figure 3. Restoration of complex IV activity by cytochrome c 1.5 h after addition of apoptosis inducers actinomycin D (*a*), H7 (*b*), and daunorubicin (*c*). Each arrow shows the addition of 10 µM cytochrome c. Values are means (SDs are smaller than the symbols), and asterisks indicate a significant difference from the activity of the control run at each time point (ANOVA Bonferroni/Dunn procedure, and **$P < 0.01$).

When catalase was added to HL-60 cells with actinomycin D, the activity of complex II was significantly restored to 93 ± 13% of the control at 1.5 h. Catalase also significantly raised the activity of complex IV at 0.5, 1, and 1.5 h (fig. 5). These observations indicate that hydrogen peroxide is generated and causes the release of cytochrome c during apoptosis caused by actinomycin D.

Apoptosis of HL-60 cells by H7

H7, an inhibitor of protein kinase C, has been reported to cause apoptosis in HL-60 cells [21, 22], although the mechanism has not been clarified. The activity of caspase-3-like protease increased significantly ($P < 0.01$) from 0.073 ± 0.075 U at the start to 0.90 ± 0.35 U, 2 h after the addition of H7 and reached a maximum level after 4 h (1.65 ± 0.18 U) (fig. 1b). After 6 h, enzyme activity was 1.46 ± 0.24 U. These results demonstrate that apoptosis caused by H7 also involves the activation of caspase-3.

Catalase significantly inhibited the activation of caspase-3 by H7 after 4 h (0.79 ± 0.16 U) and 6 h (0.58 ± 0.16 U) (fig. 1b). These results indicate that hydrogen peroxide, a mediator of apoptosis of HL-60 cells induced by H7 [12], is also involved in the activation of caspase-3.

PBN at 20 mM significantly inhibited the activation of caspase-3 4 h (0.57 ± 0.27 U), and 6 h (0.45 ± 0.21 U) after the addition of H7 (fig. 1b). These results suggest that hydroxyl radicals are also involved in the activation of caspase-3 during apoptosis of HL-60 cells caused by H7.

The apoptotic rate of HL-60 cells induced by H7 after 6 h was 67.4 ± 10.7%, which was significantly reduced to 44.9 ± 2.3% and 31.8 ± 9.3% by catalase and PBN, respectively. These results were consistent with our previous study [12].

The effect of H7 on mitochondrial activity was also estimated based on oxygen consumption (fig. 2c). One and 1.5 h after the addition of H7 to HL-60 cells, the activities of complexes I and IV were significantly depressed compared to the control values. The activity of complex IV at 1.5 h (19 ± 5% of the control) was restored significantly to 81 ± 11% of the control by the addition of 10 µM of cytochrome c (fig. 3b). The restored value was not significantly different from that of the control.

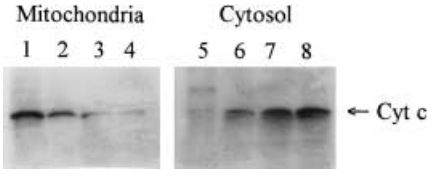

Figure 4. Analysis of cytochrome c, 1.5 h after addition of apoptosis inducers. Cytochrome c (arrow) was analyzed by Western blot as described in the text. Mitochondrial fractions: lane 1, control; lane 2, actinomycin D; lane 3, H7; lane 4, daunorubicin; and cytosolic fractions: lane 5, control; lane 6, actinomycin D; lane 7, H7; lane 8, daunorubicin.

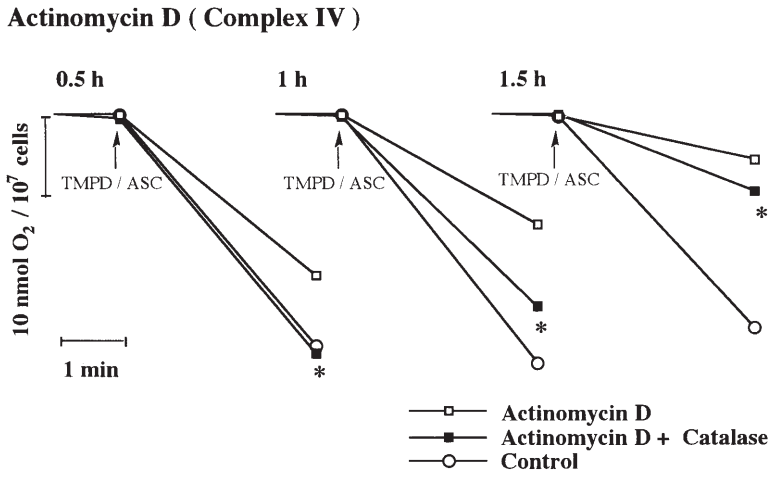

Figure 5. Activities of complex IV in HL-60 cells, 0.5, 1, and 1.5 h after no additions (Control), addition of actinomycin D, and addition of actinomycin D + catalase. Values are means (SDs are smaller than the symbols), and asterisks (actinomycin D + catalase) indicate a significant difference ($P < 0.05$) from the activity of complex IV in cells with only actinomycin D added (ANOVA Bonferroni/Dunn procedure.

Therefore, the activity of complex IV was again assumed to be almost completely restored. Release of cytochrome c from mitochondria to the cytosol 1.5 h after the addition of H7 was also demonstrated by Western blot analysis (fig. 4). These results show clearly that the release of cytochrome c caused the inactivation of complex IV, and cytochrome oxidase seems to remain intact 1.5 h after the addition of H7. This observation is also consistent with caspase-3 activation starting approximately 2 h after the release of cytochrome c (fig. 1b).

When catalase was added to HL-60 cells together with H7, the activity of complex IV was significantly restored to 31 ± 7% of the control at 1.5 h. These observations demonstrate that hydrogen peroxide is generated and causes the release of cytochrome c from mitochondria during apoptosis caused by H7. The depression of complex II was not recovered by catalase.

Apoptosis of HL-60 cells by daunorubicin

The anthracycline daunorubicin is widely used in the treatment of acute myeloid leukemia and has been reported to cause apoptosis in HL-60 cells [16, 23–26] through ceramide generation [17, 23] and activation of NF-κB, which may involve ROS [24]. The activity of caspase-3-like protease increased significantly ($P < 0.01$) from 0.073 ± 0.075 U at the start to 1.02 ± 0.13 U 2 h after the addition of daunorubicin and reached a maximum evel after 4 h (1.62 ± 0.50 U) (fig. 1c). After 6 h, the enzyme activity was 1.20 ± 0.31 U. These results demonstrate that apoptosis caused by daunorubicin also involves the activation of caspase-3.

Catalase significantly inhibited the activation of caspase-3 by daunorubicin after 4 h (0.68 ± 0.11 U) and 6 h (0.54 ± 0.14 U) (Fig. 1c). These results indicate that hydrogen peroxide, a mediator of apoptosis of HL-60 cells induced by daunorubicin [12], is also involved in the activation of caspase-3.

PBN significantly inhibited caspase-3 activation 4 h (0.57 ± 0.16 U) and 6 h (0.44 ± 0.11 U) after the addition of daunorubicin (fig. 1c). These results suggest that hydroxyl radicals are also involved in the activation of caspase-3 during apoptosis of HL-60 cells induced by daunorubicin. The apoptotic rate of HL-60 cells by daunorubicin after 6 h was 74.9 ± 2.8%, which was reduced significantly to 35.7 ± 5.1% and 26.7 ± 6.6% by catalase and PBN, respectively. These results were consistent with our previous report [12].

The effect of daunorubicin on mitochondrial activity was also estimated based on oxygen consumption (fig. 2d). At 1.5 h after the addition of H7 to HL-60 cells, the activities of complexes I and IV were significantly depressed compared to the control values. The activity of complex IV after 1.5 h (28 ± 18% of the control) was significantly restored to 89 ± 14% by 10 µM of cytochrome c (fig. 3c). The restored value was not significantly different from that of the control. Therefore, the activity of complex IV was again assumed to be fully restored. Release of cytochrome c from mitochondria to the cytosol 1.5 h after the addition of daunorubicin was also demonstrated by Western blot analysis (fig. 4). These results show that cytochrome c release caused the inactivation of complex IV, and cytochrome oxidase remains intact 1.5 h after the addition of daunorubicin. This observation is also consistent with the result that the activation of caspase-3 started approximately 2 h after the release of cytochrome c (Fig. 1c).

When catalase was added with daunorubicin, complex IV inhibition was not reversed at 1.5 h. This observation suggests that the action of hydrogen peroxide induced by

daunorubicin is somewhat different from that in the cases of actinomycin D and H7.

In conclusion, the present inhibition experiments using catalase and a spin trap, PBN, indicate that hydrogen peroxide and the hydroxyl radical are common mediators in the activation of caspase-3 during apoptosis of HL-60 cells caused by drugs such as actinomycin D, H7, or daunorubicin, which have apparently different mechanisms of action. We also confirmed the release of cytochrome c from mitochondria by direct measurement of cytochrome c and complex IV activity. Complex IV activity of HL-60 cells was depressed by treatment with actinomycin D, H7, or daunorubicin, and was fully restored by the addition of cytochrome c. In addition, the suppression of complex IV by actinomycin D and H7 was inhibited by the presence of catalase. These results indicate a direct relationship between the generation of hydrogen peroxide and the release of cytochrome c causing the activation of caspase-3 during apoptosis induced by actinomycin D, H7, and daunorubicin.

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