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Comparison of the Apoptotic Pathways Induced by L-Amino Acid Oxidase and Hydrogen Peroxide

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We have found that L-amino acid oxidase (LAO) induces apoptosis in several cultured cell lines by generating H_2O_2 [Suhr, S.M. and Kim, D.S. (1996) *Biochem. Biophys. Res. Commun.* 224, 134-139]. It is demonstrated in the present work that the LAO-induced apoptotic mechanism is clearly distinguished from the one stimulated directly by exogenous H_2O_2. MOLT-4 cells undergo somewhat different morphological changes depending on the apoptotic inducer, LAO or H_2O_2. LAO-induced apoptosis can be protected by the antioxidant *N*-acetylcysteine or the free radical scavenger melatonin, while H_2O_2-induced apoptotic cell death is not protected. A caspase inhibitor, acetyl-Tyr-Val-Ala-Asp-aldehyde (ac-YVAD-aldehyde), prevents cell death when the apoptosis is induced by exogenous H_2O_2. On the other hand, the ac-YVAD-aldehyde tetrapeptide inhibitor that is dominantly effective on interleukin-1β converting enzyme failed to block the apoptotic event initiated by LAO. Several lines of experimental evidence suggest that apoptotic cell death induced by LAO is not due solely to the hydrogen peroxide produced by the enzymatic reaction.

Key words: apoptosis, hydrogen peroxide, L-amino acid oxidase, snake venom.

Hydrogen peroxide-induced DNA damage and cell death have been attributed to the direct cytotoxicity of H_2O_2 and other reactive oxygen species generated from H_2O_2 (*1*). Hydrogen peroxide causes DNA double strand breaks (*2*) because it is stable and able to freely penetrate the cell, thereby gaining access to DNA. Hydrogen peroxide causes cell death *via* the oxidation of membrane poly-unsaturated fatty acids to fatty acid hydroperoxides (*3*). Predominant processes resulting from oxidative stress include membrane lipid breakdown, the loss of intracellular calcium levels, the formation of reactive oxygen species (ROS), and alterations in metabolic pathways. Ceramide degraded from membrane lipids is a potential mediator of signal transduction (*4, 5*). Furthermore, there is evidence that ceramide signals activate the ICE/CED-3 effector system to execute apoptosis (*6*). Recent investigations have identified at least ten distinct human caspase genes with different substrates (*7-10*). This means that caspases might be synthesized or activated in a tissue-specific manner and that different caspases might respond to different stimuli (*11-13*). Selenium-dependent glutathione peroxidase plays a protective role in oxidative stress-induced apoptosis (*14, 15*), and various antioxidants such as *N*-acetylcysteine (NAC) and free radical scavengers prevent ROS-induced cell death (*16, 17*). In our previous report, we clearly demonstrated that snake venom-derived LAO is responsible for inducing apoptotic cell death by increasing local concentrations of H_2O_2 around the cultured cell membrane (*18*). The fact that Trolox, a membrane antioxidant, inhibits LAO-induced apoptosis implies that membrane oxidation by H_2O_2 resulting from the LAO enzymatic reaction is responsible for the induction of apoptosis (*19*). In the present study, the efficiency of several antioxidants in preventing apoptosis was observed and the intracellular responses to apoptotic cell death induced by LAO or H_2O_2 were investigated by flow cytometry analysis.

MATERIALS AND METHODS

Cell Culture and Treatments—MOLT-4 (human T-cell acute lymphoblastic leukemia) cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) and supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 0.1 mg streptomycin at 37°C in a 5% CO_2-humidified incubator. For time course studies, cells (10^6/ml) in 96-well microtiter plates were treated with 200 nM LAO purified from Korean snake (*Agkistrodon halys*) venom (*18*) or with 0.25 mM H_2O_2. Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazollium bromide] assay (*20*). The resulting formazan product was resolved in 0.5 N HCl, 5% isopropanol, and measured spectrophotometrically at 570 nm.

DNA Analysis—Cells were pelleted by centrifugation for

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Abbreviations: ac-YVAD-aldehyde, acetyl-Tyr-Val-Ala-Asp-aldehyde; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazollium bromide; H_2O_2, hydrogen peroxide; ICE, interleukin-1β converting enzyme; LAO, L-amino-acid oxidase; NAC, *N*-acetylcysteine; ROS, reactive oxygen species.

10 min at 4°C, rinsed once with ice-cold PBS, and resuspending in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.1% Triton X-100. Proteinase K (100 μg/ml) was added, and the lysate was incubated overnight at 50°C. RNase A then added and the lysates were incubated at 37°C for 30 min. Fragmented DNA was extracted twice with phenol, once with chloroform, and precipitated in ethanol with 0.5 M ammonium acetate. The fragmented DNA was collected by centrifugation at 15,000$\times g$ for 20 min, washed with 70% ethanol, and electrophoresed in 1.5% agarose gels. Separated DNA bands were visualized under UV light after staining with ethidium bromide.

Measurement of Intracellular Peroxides—Intracellular peroxide levels were measured using an oxidation-sensitive fluorescent probe, 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) (*21*). In the presence of various intracellular peroxides, 2′,7′-dichlorofluorescein (DCFH) is oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Peroxide levels were assessed by the method described previously (*22*). MOLT-4 cells (5×10^5/ml) were incubated for 1 h at 37°C with 5 μM DCFH-DA (Molecular Probes, Eugene, OR) dissolved in DMSO. Cells were washed with culture medium to remove the remaining fluorescent probe and treated with an apoptosis-inducing agent. After treatment, the cells were harvested by centrifugation for 10 min at 4°C. Peroxide levels were measured using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively. The appearance of intracellular peroxide was also observed under a fluorescence microscope.

Protection from Apoptotic Cell Death—*N*-Acetylcysteine (NAC) and melatonin were used as free radical scavengers. MOLT-4 cells (10^5 cells) were plated into individual wells of a 96-well plate. Prior to the addition of 200 nM LAO or 0.25 mM H_2O_2, the cells were pre-incubated for 1 h with NAC (1 mM), melatonin (0.2 mM), or ICE-inhibitor ac-YVAD-aldehyde dissolved in PBS. Eighteen hours after the addition of the apoptotic inducer, cell viability was determined by the trypan blue exclusion test (*23*) or the MTT assay (*20*).

Cell Cycle Analysis by Flow Cytometry—Cells were grown for 2 days to 80% confluence and synchronized with aphidicolin (1 μg/ml). After 12 h, the cells were released from the aphidicolin-block by washing twice with RPMI-1640 medium. The cells were harvested at various times after treatment with LAO, washed once with PBS, and fixed overnight at −20°C after the gradual addition of 70% cold ethanol. The cells were washed again and resuspended in 500 μl of PBS to make 10^6 cells/ml. The cells were then incubated at 37°C for 30 min with RNase A (10 μg/ml) and then stained with propidium iodide (5 μg/ml) for 30 min at room temperature (*24*). The cell cycle was analyzed with a fluorescence-activated cell sorter (Becton Dickinson).

RESULTS AND DISCUSSION

We previously demonstrated that the LAO purified from Korean snake (*Agkistrodon halys*) venom induces apoptotic cell death by generating hydrogen peroxide as a reaction product in MOLT-4 cell culture (*18*). In order to examine whether the LAO-induced apoptotic process depends entirely on the production of H_2O_2, we have attempted to study the effect of exogenous H_2O_2 (0.25 mM)- or LAO (200 nM)-induced MOLT-4 cell death from several aspects. Agarose gel electrophoresis of DNA isolated from LAO-treated MOLT-4 cells showed a distinct ladder-like fragmentation pattern for cells treated with H_2O_2 (Fig. 1). This shows that more extensive DNA fragmentation takes place in LAO-induced apoptosis than in H_2O_2-induced apoptosis where large DNA fragments remain in the top region of the gel (Fig. 1). The high molecular weight DNA (Fig. 1, lane 1) was not further digested when the cells were treated with higher concentrations of H_2O_2 up to 0.5 mM (data not shown). It is generally accepted that cells undergo necrosis rather than apoptosis in the presence of excess amounts of hydrogen peroxide (*25, 26*). The observed morphological features were also different in the apoptotic cell death induced by LAO and H_2O_2. MOLT-4 cells showed blebbing of the cell membrane without changes in cell size.

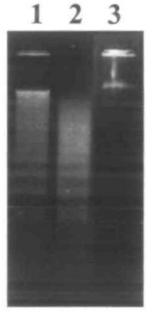


Fig. 1. **DNA fragmentation induced by H_2O_2 or LAO in MOLT-4 cells.** DNA was isolated from MOLT-4 cells (10^6/ml) treated for 18 h with 0.25 mM H_2O_2 (lane 1) or 200 nM LAO (lane 2), and then analyzed by 1.5% agarose gel electrophoresis. Lane 3 is control DNA isolated from untreated cells.

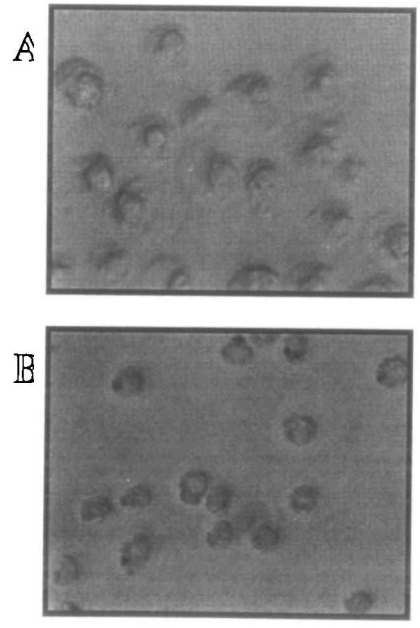


Fig. 2. **Microscopic observation of MOLT-4 cells treated with H_2O_2 or LAO.** MOLT-4 cells were treated with 0.25 mM H_2O_2 (A) or 200 nM LAO (B) for 18 h, and then observed under an inverted phase contrast microscope.

However, LAO-treated cells showed reduced cell volume due to the removal of apoptotic bodies (Fig. 2).

We used a fluorescent probe to detect intracelluar levels of reactive oxygen intermediates in cultured MOLT-4 cells. The fluorescent probe DCFH-DA is a suitable agent for detecting intracellular peroxide species (27). DCFH-DA is freely permeable across cell membranes and is incorporated into hydrophobic lipid regions of the cells. Esterases in the cells cleave off the acetate moiety of the fluorescent probe to form DCFH, which is nonfluorescent. Subsequently, hydrogen peroxide and peroxides produced by the cells oxidize DCFH to fluorescent DCF. In the present work, the accumulation of intracellular peroxides occurred at different stages of apoptosis depending on the apoptotic inducer. When cells were incubated with DCFH-DA prior to the initiation of apoptosis, the oxidation of nonfluorescent DCFH to fluorescent DCF was detected 45 m after apoptotic induction by H_2O_2 (Fig. 3A). However, the appearance of fluorescence was observed in 12 h when LAO induced the apoptosis (Fig. 3B). The results of flow cytometric analysis suggest that the processes that generate intracellular peroxides are different in these two apoptotic systems. The microscopic observations also support this hypothesis (data not shown). Further investigations were carried out to assess the correlation between free radical generation and cellular damage. We have examined the effect of several antioxidants on apoptotic cell death induced by LAO or exogenous H_2O_2. Since antioxidants interfere with the MTT assay, trypan blue exclusion and manual cell counting methods were employed (23). The glutathione precursor, *N*-acetylcysteine (NAC) provided significant protection (>70%) against apoptosis in LAO-induced MOLT-4 cells, while antioxidants were much less potent in restoring viability to cells treated with H_2O_2 (Fig. 4A). Melatonin, a

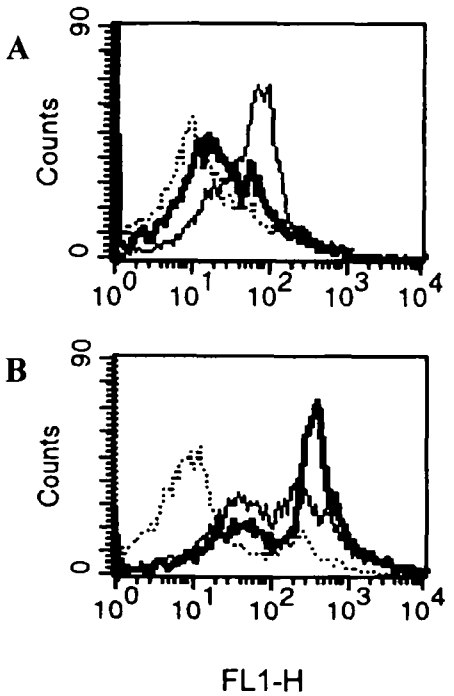


Fig. 3. **Intracellular peroxide production in MOLT-4 cells.** MOLT-4 cells (5×10^5/ml) were incubated with 5 μM DCFH-DA for 1 h at 37°C. The cells were then not treated (dotted line) or treated with apoptosis agents [0.25 mM H_2O_2 (thin line) or 200 nM LAO (thick line)]. Peroxide levels were measured after incubation for 45 min (panel A) or 12 h (panel B) using a Becton Dickinson FACScan flow cytometer with excitation and emission settings of 488 nm and 530 nm, respectively.

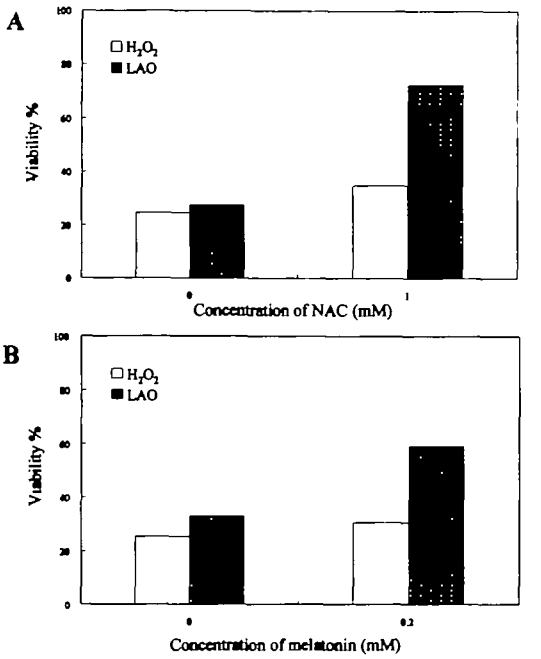


Fig. 4. **Protection against apoptosis induced by free radical scavengers.** MOLT-4 cells (10^5 cells) were plated into individual wells of a 96-well plate. Cells were pre-incubated with 1 mM NAC (A) or 0.2 mM Melatonin (B) for 1 h prior to the addition of 200 nM LAO or 0.25 mM H_2O_2 to the culture medium. Eighteen hours after the addition of an apoptotic inducer, cell viability was tested by the trypan blue exclusion test. Each analysis represents the average of three determinations.

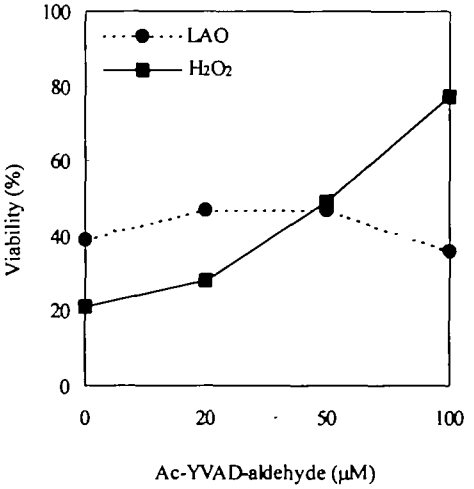
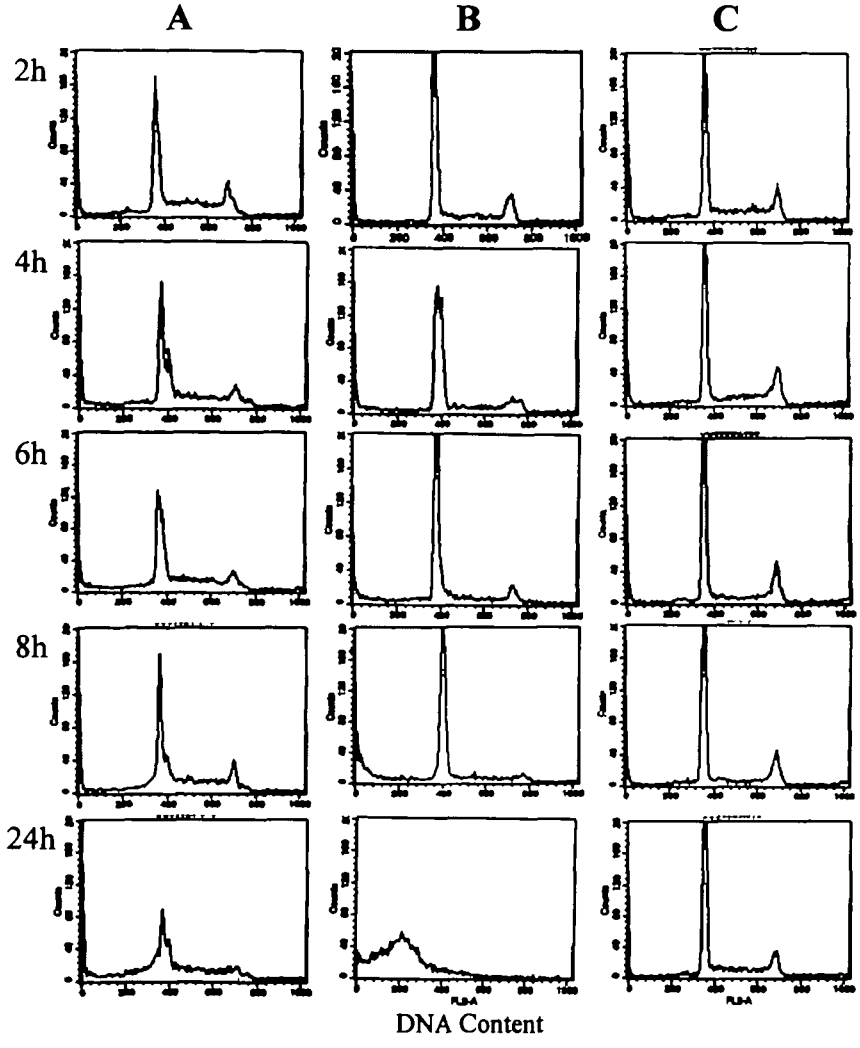


Fig. 5. **Protection against apoptosis induced by caspase inhibitor, ac-YVAD-aldehyde.** Cells (10^6/ml) were pre-incubated with ac-YVAD-aldehyde for 1 h prior to treatment with 200 nM LAO (●) or 0.25 mM H_2O_2 (■). Cell viability was determined after 18 h incubation by the MTT assay. Each analysis represents the average of three determinations.

free radical scavenger, was less effective than NAC in protecting cells from apoptotic progression (Fig. 4B). These data suggest that the apoptotic cell death induced by H_2O_2 or LAO is caused mainly by increased intracellular levels of ROS. Nevertheless, intracellular ROS levels rose much later in the apoptotic pathway in LAO-induced cells than in cells treated with H_2O_2 (Fig. 3). It is possible to hypothesize that LAO-induced apoptosis is partly mediated by intracellular ROS in addition to the extracellular H_2O_2 produced by the catalytic reaction of LAO.

In order to examine whether a common pathway of apoptosis is shared by cells induced with different agents, we investigated the susceptibility of apoptotic cells to the reversible ICE-inhibitor, ac-YVAD-aldehyde, which is known to strongly inhibit caspase 1 (*12*). Ac-YVAD-aldehyde is not effective in inhibiting ICE/CED-3 below a level of 20 μM because its permeability across cell membranes is not favorable. It has been established that various caspases are activated depending on the apoptotic stimuli (*28*). As illustrated in Fig. 5, apoptosis induced by H_2O_2 is effectively prevented by the ICE-inhibitor, and its effect was measured proportional to the inhibitor concentration. On the other hand, no such inhibition was observed in the case of LAO-induced apoptosis. These results also strengthen



	H_2O_2			LAO			Control		
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
2h	46.34	41.94	11.73	72.18	20.41	7.72	47.65	41.05	11.30
4h	45.15	54.40	0.44	74.96	18.14	6.90	56.11	33.58	10.31
6h	49.61	49.97	0.12	75.42	21.08	3.50	59.36	26.46	14.18
8h	39.51	51.32	9.17	84.07	5.91	0.12	67.41	18.58	14.01

Fig. 6. **Cell cycle analysis by flow cytometry.** MOLT-4 cells (10^6/ml) were synchronized by treatment with aphidicolin for 12 h, and released in culture medium for 12 h. Cells were treated with inducers of apoptosis, harvested at the indicated times, stained with propidium iodide, and analyzed with a Becton Dickinson flow cytometer. MOLT-4 cells were treated with 0.25 mM H_2O_2 (column A), 200 nM LAO (column B), or left untreated (column C). The results shown are representative of three separate experiments.

the possibility that the LAO-induced apoptotic pathway differs from the H_2O_2-induced case. Flow cytometry studies were carried out in order to analyze the cell cycle of apoptotic MOLT-4 cells treated with LAO or H_2O_2. In comparison to control cells, MOLT-4 cells cultured in the presence of LAO were arrested in G1 phase at 6 h, and entry into S phase was delayed (Fig. 6). The typical apoptotic cell peak appears 24 h later, as shown in the flow cytometry analysis data. On the other hand, H_2O_2-treated cells exhibited much more accelerated entry into S phase in 2 h followed by apoptotic cell death in 6 h without progression into G2/M (Fig. 6). From the results obtained by flow cytometry, it is reasonable to conclude that the LAO-induced apoptotic pathway differs from the one induced by exogenously added H_2O_2 to the culture of MOLT-4 cells. These results further support the hypothesis that the LAO-induced apoptosis can not be explained simply by the effect of H_2O_2.

Based on the data presented in this communication, as well as previous evidence, of LAO binding to the cell surface (*18*), we have clearly demonstrated that the LAO-induced apoptotic event does not depend solely on H_2O_2, which is produced by the catalytic activity of LAO. It will be worthwhile to investigate the molecular details of which intracellular components are specifically associated with LAO-induced apoptosis.

REFERENCES

1. Ueda, N. and Shah, S.V. (1992) Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J. Clin. Invest.* **90**, 2593-2597
2. Schraufstatter, I., Hyslop, P.A., Jackson, J.H., and Cochrane, C.G. (1988) Oxidant-induced DNA damage of target cells. *J. Clin. Invest.* **82**, 1040-1050
3. Halliwell, B. and Gutteridge, J.M. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **210**, 1-14
4. Westwick, J.K., Bielawska, A.E., Dbaibe, G., Hannum, Y.A., and Brennerm, D.A. (1995) Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* **270**, 22689-22692
5. Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R.N. (1996) Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. *Nature* **380**, 75-79
6. Ghayur, T., Hugunin, M., Talanian, R.V., Ratnofsky, S., Quinlau, C., Emoto, Y., Pandey, P., Patta, R., Hyang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) Proteolytic activation of protein kinase C-δ by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med.* **184**, 2399-2404
7. Bing, A. and Dou, Q.P. (1996) Cleavage of retinoblastoma protein during apoptosis: An interleukin 1B-converting enzyme-like protease candidate. *Cancer Res.* **56**, 438-442
8. Nicholson, D.W. and Thornberry, N.A. (1997) Caspase: killer proteases. *Trends Biochem. Sci.* **22**, 299-306
9. Janicke, R.U., Walker, P.A., Lin, X.Y., and Porter, A.G. (1996) Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J.* **15**, 6969-6978
10. Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesu, G., Thornberry, N.A., Wong, W.W., and Yuan, J. (1996) Human ICE/ CED-3 protease nomenclature. *Cell* **87**, 171
11. Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997) Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16**, 2271-2281
12. Villa, P., Kaufmann, S.H., and Earnshow, W.C. (1997) Caspases and caspase inhibitors. *Trends Biochem. Sci.* **22**, 388-393
13. Janicke, R.U., Sprengart, M.L., Wati, M.R., and Porter, A.G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J. Biol. Chem.* **273**, 9357-9360
14. Kayanoki, Y., Fujii, J., Islam, K.N., Suzuki, K., Kawata, S., Matsuzawa, Y., and Taniguchi, N. (1996) The protective role of glutathione peroxidase in apoptosis induced reactive oxygen species. *J. Biochem.* **119**, 817-822
15. Wu, L., Lanfer, J., and Harrison, P.R. (1995) The selenium metabolite selenodiglutathione induces cell death by a mechanism distinct from H_2O_2 toxicity. *Carcinogenesis* **16**, 1579-1584
16. Shaikh, A.Y., Xu, J., Wu, Y., He, L., and Hsu, C.Y. (1997) Melatonin protects bovine cerebral endothelial cells from hyperoxia-induced DNA damage and death. *Neurosci. Lett.* **229**, 193-197
17. Reiter, R.J., Carneiro, R.C., and Oh, C.S. (1997) Melatonin in relation to cellular antioxidative defense mechanisms. *Horm. Metab. Res.* **29**, 363-372
18. Suhr, S.-M. and Kim, D.-S. (1996) Identification of the snake venom substance that induces apoptosis. *Biochem. Biophy. Res. Commun.* **224**, 134-139
19. Torii, S., Naito, M., and Tsuruo, T. (1997) Apoxin-I a novel apoptosis-inducing factor with l-amino acid oxidase activity purified from western diamondback rattle snake venom. *J. Biol. Chem.* **272**, 9539-9542
20. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63
21. Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C., and Thomas, M. (1983) Flow cytometric studies of oxidative product formation by neutrophils: A grades response to membrane stimulation. *J. Immunol.* **130**, 1910-1917
22. Hokenbery, D.M., Oltivai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251
23. Behl, C., Davis, J.B., Lesley, R., and Schubert, D. (1994) Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* **77**, 817-827
24. Jayadev, S., Liu, B., Bielawska, A.E., Lee, J.Y., Nazaire, F., Pushkareva, M.Y., Obeid, L.M., and Hannun, Y.A. (1995) Role for ceramide in cell cycle arrest. *J. Biol. Chem.* **270**, 2047-2052
25. Sakamoto, T., Repasky, W.T., Uchida, K., Hirata, A., and Hirata, F. (1996) Modulation of cell death pathways to apoptosis and necrosis of H_2O_2-treated rat thymocytes by lipocortin1. *Biochem. Biophy. Res. Commun.* **220**, 643-647
26. Hampton, M.B. and Orrenius, S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implications of apoptosis. *FEBS Lett.* **414**, 552-556
27. Gorman, A.M., Samali, A., MaGowan, A.J., and Cotter, G. (1997) Use of flow cytometry techniques in studying mechanisms of apoptosis in leukemic cells. *Cytometry* **29**, 97-105
28. Kheaume, E., Cohen, L.Y., Uhlmann, F., Lazure, C., Alam, A., Hurwitz, J., Sekaly, R.P., and Denis, F. (1997) The large subunit of replication factor C is a substrate for caspase-3 in vitro and is cleaved by a caspase-3 like protease during Fas mediated apoptosis. *EMBO J.* **16**, 6346-6354