The Journal of Applied Pharmacology, 13, 207-213(2005)

Glucose Oxidase/glucose Induces Apoptosis in C6 Glial Cells via Mitochondria-dependent Pathway

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(Received June 29, 2005; Accepted August 16, 2005)

Abstract – It has been proposed that reactive oxygen species (ROS), mainly superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), may mediate oxidative stress. Production of H_2O_2 during oxidative phosphorylation, inflammation, and ischemia can cause oxidative stress leading to cell death. Although glucose oxidase (GOX) in the presence of glucose continuously generates H_2O_2, it is not clear whether GOX produces apoptotic cell death in C6 glial cells. Thus, we investigated the mechanism by which GOX induces cell death. Cells were incubated with different concentration of GOX in the presence of glucose where cell viability, TUNEL and DNA ladder were analyzed. Results indicated that GOX exhibited cytotoxicity in a dose dependent manner by MTT assay. TUNEL positive cell and DNA laddering showed that GOX-induced cytotoxicity was due to apoptosis. Western blot analysis also showed that the cleaved caspase-3 level was detected in the GOX-treated cells at 10 mU/ml and increased dramatically at 30 mU/ml. Cleaved PARP also appeared at 10 mU/ml and lasted at 20 or 30 mU/ml of GOX. Cytochrome c level was increased by GOX dose dependently, which was contrast to Bcl-2 expression level. These results suggest that GOX induces apoptosis through caspase-3 activation, which followed by cytochrome c release from mitochondria through regulating of Bcl-2 level.

Key words □ GOX, apoptosis, H_2O_2, caspase-3, PARP

INTRODUCTION

Apoptosis plays a significant role in embryonic development, tissue homeostasis and neurodegeneration (Cohen, 1991; Kerr *et al.*, 1994; Gehri *et al.*, 1996; Kusiak *et al.*, 1996; Jacobson *et al.*, 1997). Inappropriate apoptosis is involved in many disorders, including immune deficiencies such as AIDS, carcinogenesis, cancer progression, autoimmune diseases and Alzheimer's disease (AD) (Carson and Ribeiro, 1993; Barr and Tomei, 1994; Kusiak *et al.*, 1996). Therefore, understanding the molecular mechanisms that control apoptosis is important for therapeutic intervention.

It has been proposed that reactive oxygen species (ROS), mainly superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), as well as 4-hydroxynonenal (HNE), a major aldehydic end-product of lipid peroxidation, may mediate neurotoxicity (Christen, 2000; Kruman *et al.*, 1997; Mark *et al.*, 1997). Involvement of oxidative stress, including protein oxidation, lipid peroxidation, and peroxynitrite formation, has been reported in either human brain samples or in experimental models of AD (Behl *et al.*, 1994; Butterfield *et al.*, 1994; Markesbery and Carney, 1999; Smith et al., 1998). Production of H_2O_2 during oxidative phosphorylation (Boveris, 1977), inflammation (Badwey and Karnovsky, 1980), and ischemia (Cao *et al.*, 1988) can cause oxidative stress leading to cell death. In particular, H_2O_2 may induce both apoptosis and necrosis depending on the concentration of the oxidant employed and/or the type of cell being studied (Coyle and Puttfarcken, 1993; Ichas *et al.*, 1997, Hoyt *et al.*, 1997; Olanow, 1993). In cultured cortical neurons it has been demonstrated that exposure to H_2O_2 causes apoptosis (Whittemore *et al.*, 1994; 1995). ROS is known to be able to elicit apoptosis in a large variety of cultured cells of different origins (Han *et al.*, 2001; Kalinich *et al.*, 2000), including some cultured neuronal

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cells (Kaltschmidt *et al.*, 1997; Lo et al., 1996). Data reported in the last decade indicate that ROS can act as effective mediators of pathophysiological effects of oxidative stress in several chronic human diseases of relevant social impact such as atherosclerosis, liver fibrosis, and neurodegenerative disorders of the CNS (Behl *et al.*, 1992; Kunsch and Medford, 1999; Poli and Parola, 1997), to name just a few.

Induction of apoptosis by oxidative stress has been observed in hippocampal (Enokido and Hatanaka, 1993), embryonic cortical (Ratan *et al.*, 1994) and forebrain neurons (Hoyt *et al.*, 1997). Oxidative stress is a characteristic feature in a number of neurodegenerative disorders (Coyle and Puttfarcken, 1993; Olanow, 1993). A direct link between oxidative stress and apoptosis in neurodegeneration has been suggested (Gorman *et al.*, 1996).

Glucose oxidase (GOX) catalyzes the formation of gluconolactone from glucose and also forms H_2O_2 as a by-product of the reaction. The GOX enzyme binds to beta-D-glucose (an isomer of the six carbon sugar, glucose) and aids in breaking the sugar down into its metabolites. GOX is a dimeric protein which catalyzes the oxidation of beta-D-glucose into D-glucono-1,5-lactone which then hydrolyzes to gluconic acid. In order to work as a catalyst, GOX requires a cofactor, flavin adenine dinucleotide (FAD). FAD is a common component in biological oxidation-reduction (redox reactions). Redox reactions involve a gain or loss of electrons from a molecule. In the GOX catalyzed redox reaction, FAD works as the initial electron acceptor and is reduced to $FADH_2$. Then $FADH_2$ is oxidized by the final electron acceptor, molecular oxygen (O_2) which can do so because it has a higher reduction potential. O_2 is then reduced to H_2O_2. From these facts, it may be suggest that GOX can induce apoptosis through continuous generation of H_2O_2. In this study, thus, we investigated the induction of apoptosis by GOX in C6 glial cell and the signaling pathway respect to GOX-induced apoptosis.

MATERIALS AND METHODS

Materials

RPMI 1640 media, fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) were obtained from Gibco BRL (Rockville, MD). An *in Situ* Cell Death Detection Kit was obtained from Boehringer Mannheim (Mannheim, Germany). DNAzolTM genomic DNA isolation reagent was provided from iNTRON Inc., (Korea). The MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide), GOX and all other chemicals were purchased from Sigma Chemicals (St.Louis, MO). Anti-cleaved caspase-3, anti–Bcl-2, anti-PARP, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

C6 cell was obtained from Korean Cell line Bank (KCLB; Seoul, Korea). For all other experiments, C6 cells were cultured in DMEM supplemented with 5% heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The cells were seeded at a density of 1×10^6 cells/100-mm dishes and grown until 70~80% confluence. Before any experiment, cells were transferred from medium with 5% FBS to a medium with 0.5% FBS and were incubated for 1 day. Cells were incubated in a 37°C incubator, fully humidified, with 5% CO_2.

MTT assay

Cells were trypsinized, and cell concentration was assessed using a hemocytometer. Cells were diluted to 20,000 cells/100 μl in 1× RPMI 1640 with 10% FBS and then plated on a 96-well plate at 100 μl/well. The following day, the culture medium was changed to a 0.5% FBS medium. Following 8 h of treatment, 50 μl of MTT (2 mg/ml in 1 × PBS) were added to each well, and the plate was incubated for 4 h. All culture media were then removed, and the cells were resuspended in 100 μl DMSO and incubated for 1 h. Cell viability was assessed by colorimetric change using Microplate Reader at 570 nm (Bio-Tek, Winooski, VT, USA).

Analysis of DNA fragmentation

Genomic DNA was extracted from C6-cells treated with GOX for 8 h using DNAzolTM genomic DNA isolation reagent. Cells were lysed in 1 ml of DNAzol for 5 min and centrifuged at 12,000 g for 10 min. DNA was precipitated with 100% ethanol, washed twice with 95% ethanol and solubilized in TE buffer (10 mM Tris-HCl, 1 mM EDTA). Ten mg of DNA were electrophoretically separated on 2% agarose gel containing ethidium bromide. DNA was visualized on UV transluminator (Biometra TI3, USA) and gels were photographed with Polaroid camera (Fisher Scientific, USA).

TUNEL assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay was used to assess the extent of cell death in treated cells. Briefly, cells from each treatment were grown on six-well cell culture plates (Corning,

Corning, NY, USA) and were treated as described above. Following 24 h treatment, cells were washed with PBS and then sedimented onto microscopic slides. Residual PBS was then removed, and cells were fixed using 95% ethanol and allowed to dry overnight. The cells were again fixed using 4% methanol-free formaldehyde in PBS. The slides were again washed with PBS, and fragmented DNA was detected in apoptotic cells by adding fluorescein 12-dUTP to nicked ends of DNA (Apoptosis Detection System, Promega, Madison, WI, USA). Slides were incubated for 1 h at 37°C, and the reaction was terminated with 2 × NaCl and sodium citrate (2 × SSC, Promega). The slides were washed in PBS and then visualized with a fluorescent light microscope at 400 ×, and green fluorescence was correlated with DNA fragmentation. Experiments were done in triplicate, and the percentage of TUNEL-positive cells was determined.

Western blotting

The cells were harvested and lysed with a RIPA buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH7.5) and the protease inhibitors. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford. IL). 30 μg of the total protein were electrophoresed on 7.5% polyacrylamide gels, transferred to a PVDF membrane and blocked in 5% non-fat milk in 1 × TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The membrane was incubated with the primary antibodies overnight at 4°C in 5% non fat milk in TBST, washed extensively with TBST, and then incubated with 1:5000 anti-rabbit or mouse-HRP (Amersham Pharmacia Biotech, Piscataway, NJ). The signals were detected by ECL (Amersham, Piscataway, NJ).

Statistical analysis

Results were assessed using StatView 4.5 software (Abacus Concepts, Berkeley, CA, USA). Results were compared using one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) post hoc test at a 95% confidence interval. Data were presented as mean ± SEM. Significant difference from control values was indicated by $^{**}P < 0.01$.

RESULTS

The effect of GOX on the viability of C6 glial cells

The effects of GOX on the viability of C6 cells were examined using the MTT assay. After incubation of C6 cells for 8 h in the presence of 1~25 mU/ml of GOX, the viability was reduced as the concentration of GOX was increased (Fig. 1). The cells exposed to 15 mU/ml of GOX for 8 h showed prominent decrease in cell viability (viability 38.42 ± 4.2%), and almost all cells died at 25 mU/ml of GOX.

GOX induced apoptotic cell death by DNA strand break and TUNEL assay

To determine whether the cytotoxicity by GOX was related to apoptosis, we performed TUNEL assay and DNA fragmentation anaylsis. As shown in Fig. 2, GOX increased DNA strand break in a concentration dependent manner. DNA lad-

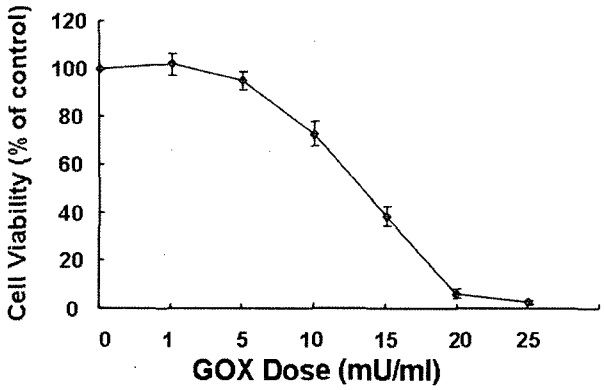

Fig. 1. Dose-dependent GOX-induced cell toxicity in C6 cells. Cells were incubated with GOX for 8 h, and cell viability was determined by MTT assay. The results are shown as a mean ± SEM of four independent experiments.

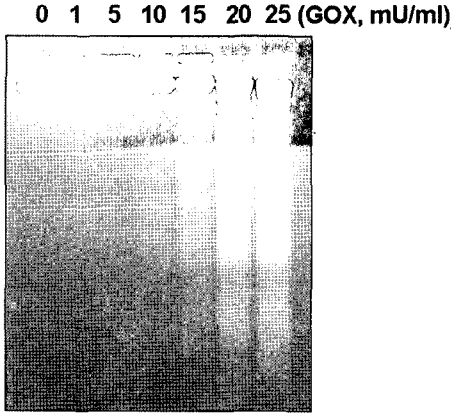

Fig. 2. GOX induces DNA fragmentation in C6 cells. GOX was treated in C6-cells for 8 h in with different doses. Lane 1, Control; Lane 2, GOX 1 mU/ml; Lane 3, GOX 5 mU/ml; Lane 4, GOX 10 mU/ml; Lane 5, GOX 15 mU/ml; Lane 6, GOX 20 mU/ml; Lane 7, GOX 25 mU/ml. Genomic DNA was extracted from C6 cells treated with GOX for 8 h using DNAzolTM genomic DNA isolation reagent. Ten μg of DNA were electrophoretically separated on 2% agarose gel containing ethidium bromide.

dering starts to appear at 10 mU/ml of GOX and increased dramatically at 20 or 25 mU/ml of GOX. TUNEL assay also showed that GOX induced apoptotic cell death in a concentration-dependent manner, especially at the dose of 20 or 25 mU/ml of GOX (Fig. 3A). Thirty mU/ml of GOX effectively induced apoptosis in C6 glial cells within 4 h after treatment (Fig. 3B). The quantitative result of apoptotic cell death is shown in Fig. 4.

Mitochondria-dependent apoptotic death by GOX

In order to confirm that GOX induces apoptosis by activating the caspase pathway, western blot analysis was performed. C6 glial cells were exposed to various dose of GOX (1~30 mU/ml) and harvested at 8 h. The dose-dependent production of the cleaved caspase-3, as an apoptosis indicator, was detected (Fig. 5A). In the GOX-treated cells, the cleaved caspase-3 level was detected at 10 mU/ml of GOX and increased dramatically at 30 mU/ml. Cleaved PARP also appeared at 10 mU/ml and lasted at 20 or 30 mU/ml.

Generally, when Bcl-2 is decreased, cytochrome c releases from mitochondrial membrane. We investigated if GOX increases cytochrome c release from mitochondria through the regulation of Bcl-2 level. Western blot analysis showed that cytochrome c level was increased by GOX dose dependently, which was contrast to Bcl-2 expression level (Fig. 5B). These results suggest that GOX releases cytochrome c release from mitochondria through regulating of Bcl-2 level, which is followed by caspase-3 activation.

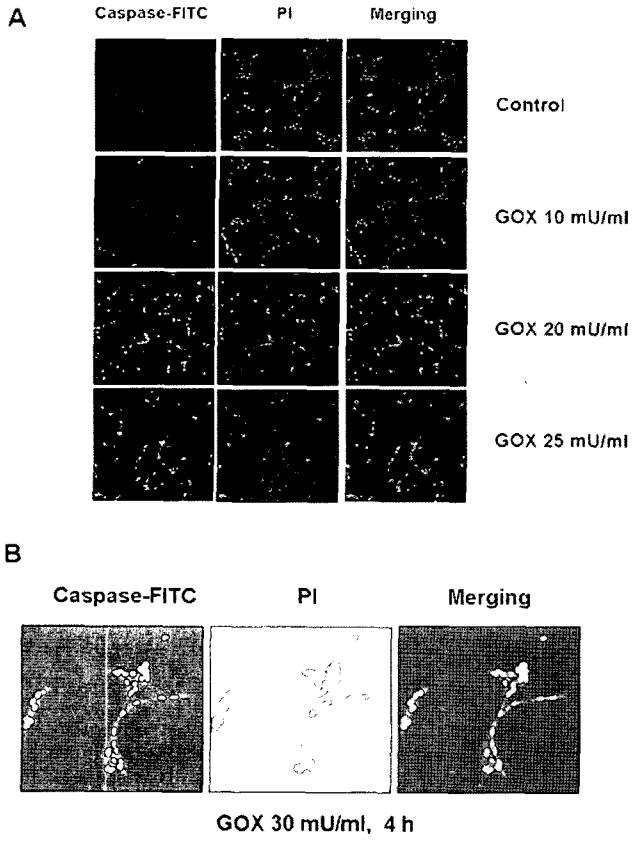

Fig. 3. TUNEL assay confirms GOX-induced apoptotic cell death. (A) C6 cells were incubated with various dose of GOX for 8 h. (B) Treatment of GOX at the dose of 30 mU/ml effectively increased apoptotic cell 4 h after treatment. Apoptotic cells were detected using by TUNEL assay as described in Method.

DISCUSSION

Apoptosis is highly involved in neurodegenerative diseases of aging, such as Parkinson's disease and AD. In Parkinson's disease, ultrastructural studies of the dopaminergic neurons revealed that they die by apoptosis. Also, an *in vitro* model of dopaminergic neuron death found that when the sphingomyelin-dependent signaling pathway is activated, these cells die by apoptosis, preceded by mitochondrial production of superoxide radicals (Ruberg *et al.*, 1997). The role of oxidative stress in Parkinson's disease can also be seen in the increased production of hydroxyl radicals from mitochondrial respiratory chain dysfunctions (Shoffner *et al.*, 1991).

Apoptosis has also been associated with AD. Oxidative stress from radical species and other apoptosis-regulating factors have been implicated in neuronal loss in AD. Many studies

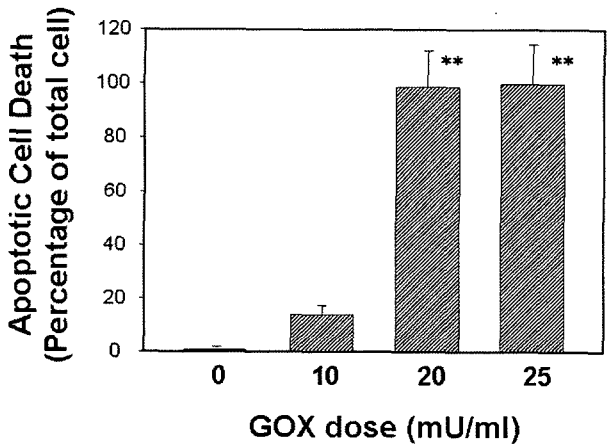

Fig. 4. Quantitative result of apoptotic cell death. Lane 1, control; Lane 2, GOX 10 mU/ml; Lane 3, GOX 20 mU/ml; Lane 4, GOX 25 mU/ml. The results are shown as a mean ± SEM of four independent experiments. Significant difference from control values was indicated by $^{**}P<0.01$.

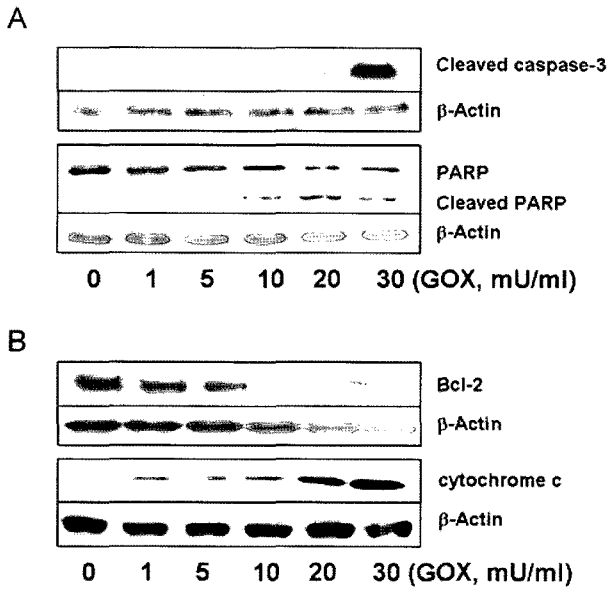

Fig. 5. Expression of apoptosis-related proteins by GOX. (A) Cleaved caspase- 3 or cleaved PARP was detected in C6 cells treated with GOX (1~30 mU/ml). (B) GOX increased cytochrome 3 level in a dose dependent manner, however, decreased Bcl-2 expression. The results were confirmed by three independent experiments.

have shown that both neurons and glial cells undergo apoptosis; in fact, the amyloid-β protein has been found to induce cultured hippocampal cells to degenerate in a process with biochemical and morphological characteristics of apoptosis (Ivins *et al.*, 1998). Moreover, the neuroprotective effects of estrogen in delaying the development of Alzheimer's disease may be due to its enhancement of the expression of the anti-apoptotic protein Bcl-X_L in cultured hippocampal neurons. This estrogen-induced enhancement of Bcl-X_L was associated with a reduction in amyloid-β-induced apoptosis, inhibiting both neurotoxicity and caspase-mediated proteolysis (Pike, 1999).

Therefore, understanding the molecular mechanisms that control apoptosis is important for therapeutic intervention in the apoptosis related neuronal diseases. In our study, we aimed to develop the effective system to mimic oxidative stress-induced apoptosis which usually happen in biological system, and to investigate the molecular mechanism involved in that system. Our results showed that GOX, which is known as continuous H_2O_2 generator, effectively induced apoptosis in C6 glial cell through mitochondria mediated pathway. GOX released cytochrome c through regulation of Bcl-2, which followed by caspase-3 activation. Our result that continuous generation of H_2O_2 using GOX system leads to an increase in activated caspase-3 level is in agreement with previous studies (D'Agnillo and Alayash, 2002). Our result is also supported by the reports that neuronal apoptosis, with the involvement of both calpains and caspases, can occur via the mitochondrial pathway, which is controlled by the Bcl-2 family proteins (Choi *et al.*, 2001). While there is more than one pathway to apoptosis, the Bcl-2 family members play a significant role in controlling apoptotic cell death in the mitochondria-mediated pathway as they are comprised of both proapoptotic members, such as Bax and Bad, and antiapoptotic members, such as Bcl-2 and Bcl-X_L (Pellegrini and Strasser, 1999).

In the vast majority of studies with H_2O_2 it was added directly to the cells as a bolus, so that cells were initially exposed to relatively high concentrations followed by a fast decrease as H_2O_2 is gradually consumed (Pugazhenthi *et al.*, 2003). Consequently, if the mode of action of H_2O_2 is concentration dependent (as most probably is the case), the results might appear inconsistent. In vivo the rate of H_2O_2 generation, although different for various kinds of cells, is continuous, with the steady state levels fluctuating at nanomolar concentrations (10^{-8} to 10^{-7} M) (Busciglio and Yankner, 1995). Hence, exposing cells to a continuous flow of H_2O_2, as opposed to bolus additions, represents a superior method of delivery that mimics physiological conditions. So the demands about in vitro model which coincide with chronic oxidative stress were increased. In this present study, to set up the model of chronic oxidative stress in glial cell, the GOX was used in C-6 cell. From our results, we can suggest that GOX-induced cell apoptosis can be used as an experimental model to study the molecular events associated with chronic oxidative stress-induced cell death in C6 glial cells.

It is now well known that mitochondria play a central regulatory role in apoptosis, particularly through the cytochrome c pathway. Also, mitochondria and radical species are intimately involved in the programmed cell death that occurs during aging and exercise. Increased oxidative stress from ROS and RNS changes the cellular redox potentials, depletes glutathione, and decreases reducing equivalents like NADP and NADPH. These intracellular changes are sufficient to induce the formation of mitochondrial permeability transition pores, leading to the subsequent release of cytochrome c and the activation of the caspase cascade. Mitochondria serve as key regulators of apoptosis via this cytochrome c-mediated pathway. Many cells activate apoptosis via the cytochrome c pathway, but they also may use pathways involving other molecules that reside in the mitochondrial intermembrane space. For example, mitochondria of some cells release procaspase-3 (Green and Reed, 1998).

Taken together, we suggest that GOX results in chronic oxidative stress through continuous generation of H_2O_2, which effectively induces apoptosis through mitochondria mediated pathway in C6 glial cells. In this study, we showed that Bcl-2-decreased and cytochrome c released activation of mitochondrial apoptotic pathway is involved in GOX-induced apoptosis in C6 glial cell for the first time.

ACKOWLEDGMENTS

This work was supported by KRF Grant (2004-005- E00058).

REFERENCES

Badwey, J. A. and Karnovsky, M. L. (1990). Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* **49**, 695-726.

Barr, P. J. and Tomei, L. D. (1992). Apoptosis and its role in human disease. *Biotechnology* **12**, 487-493.

Behl, C., Davis, J. B., Cole, G. M. and Schubert, D. (1992). Vitamin E protects nerve cells from amyloid protein toxicity. *Biochem. Biophys. Res. Commun.* **186**, 944-950.

Behl, C., Davis, J. B., Lesley, R. and Schubert, D. (1994). Hydrogen peroxide mediates amyloid protein toxicity. *Cell* **77**, 817-827.

Boveris, A. (1977). Mitochondrial production of superoxide radical and hydrogen peroxide. *Adv. Exp. Med. Biol.* **78**, 67-82.

Busciglio, J. and Yankner, B. A. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature* **378**, 776-779.

Butterfield, D. A., Hensley, K., Harris, M., Mattson, M. P. and Carney, J. (1994). Beta-amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **200**, 710-715.

Cao, W., Carney, J. M., Duchon, A., Floyd, R. A. and Chevion, M. (1988). Oxygen free radical involvement in ischemia and reperfusion injury to brain. *Neurosci. Lett.* **88**, 233-238.

Carson, D. A. and Ribeiro, J. M. (1993). Apoptosis and disease. *Lancet* **341**, 1251-1254.

Choi, W. S., Lee, E. H., Chung, C. W., Jung, Y. K., Jin, B. K., Kim, S. U., Oh, T. H., Saido, T. C. and Oh, Y. J. (2001). Cleavage of Bax is mediated by caspase-dependent or -independent calpain activation in dopaminergic neuronal cells: protective role of Bcl-2. *J. Neurochem.* **77**, 1531-1541.

Christen, Y. (2000). Oxidative stress and Alzheimer's disease. *Am. J. Clin. Nutr.* **71**, 621S–629S.

Cohen, J. J. (1991). Programmed cell death in the immune system. *Adv. Immunol.* **50**, 55-85.

Coyle, J. T. and Puttfarcken, P. (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689-695.

D'Agnillo, F. and Alayash, A. I. (2002). A role for the myoglobin redox cycle in the induction of endothelial cell apoptosis. *Free Radic. Biol. Med.* **33**, 1153-1164.

Enokido, Y. and Hatanaka, H. (1993). Apoptotic cell death occurs in hippocampal neurons cultured in a high oxygen atmosphere. *Neuroscience* **57**, 965-972.

Gehri, R., Hahn, S., Rothen, M., Steuerwald, M., Nuesch, R. and Erb, P. (1996). The Fas receptor in HIV infection: expression on peripheral blood lymphocytes and role in the depletion of T cells. *AIDS* **10**, 9-16.

Gorman, A. M., McGowan, A., O'Neill, C. and Cotter, T. (1996). Oxidative stress and apoptosis in neurodegeneration. *J. Neurol. Sci.* **139**, S45-52.

Green, D. R. and Reed, J. C. (1998). Mitochondria and apoptosis. *Science* **281**, 1309-1312.

Han, H., Wang, H., Long, H., Nattel, S. and Wang, Z. (2001). Oxidative preconditioning and apoptosis in L-cells: roles of protein kinase B and mitogen-activated protein kinases. *J. Biol. Chem.* **276**, 26357-26364.

Hoyt, K. R., Gallagher, A. J., Hastings, T. G. and Reynolds, I. J. (1997). Characterization of hydrogen peroxide toxicity in cultured rat forebrain neurons. *Neurochem. Res.* **22**, 333-340.

Ichas, F. , Jouaville, L. S. and Mazat, J. P. (1997). Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* **89**, 1145-1153.

Ivins, K. J., Bui, E. T. and Cotman, C. W. (1998). Beta-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis. *Neurobiol. Dis.* **5**, 365-378.

Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.

Kalinich, J. F., Ramakrishnan, R., McClain, D. E. and Ramakrishnan, N. (2000). 4-Hydroxynonenal, an end product of lipid peroxidation, induces apoptosis in human leukemic T- and B-cell lines. *Free Radicals Res.* **33**, 349-358.

Kaltschmidt, B., Uherek, J. K., Volk, B., Bauerle, P. A. and Kaltschmidt, C. (1997). Transcription factor NF-kB is activated in primary neurons by amyloid b peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **94**, 2642-2647.

Kerr, J. F., Winterford, C. M. and Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* **73**, 2013-2026.

Kruman, I., Bruce-Keller, A. J., Bredesen, D., Waeg, G. and Mattson, M.P. (1997). Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* **17**, 5089-5100.

Kunsch, C. and Medford, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circ. Res.* **85**, 753-766.

Kusiak, J. W., Izzo, J. A. and Zhao, B. (1996). Neurodegeneration in Alzheimer disease. Is apoptosis involved? *Mol. Chem. Neuropathol.* **28**, 153-162.

Lo, Y. Y. C., Wong, J. M. S. and Cruz, T. F. (1996). Reactive oxygen species mediate cytochine activation of c-jun NH_2-terminal kinases. *J. Biol. Chem.* **271**, 15703-15707.

Mark, R. J., Lovell, M. A., Markesbery, W. R., Uchida, K. and Mattson, M. P. (1997). A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal cell death induced by amyloid b-peptide. *J. Neurochem.* **68**, 255-264.

Markesbery, W. R. and Carney, J. H. (1999). Oxidative alterations in Alzheimer's disease. *Brain Pathol.* **9**, 133-146.

Olanow W. A. (1993). radical hypothesis for neurodegeneration. *Trends Neurosci.* **16**, 439-444.

Peden, D. B., Dailey, L., DeGraff, W., Mitchell, J. B., Lee, J. G., Kaliner, M. A. and Hohman, R. J. (1994). Hydrogen peroxide effects on rat mast cell function. *Am. J. Physiol.* **267**, L85-L93.

Pellegrini, M. and Strasser, A. (1999). A portrait of the bcl-2 pro-

tein family: life, death, and the whole picture. *J. Clin. Immunol.* **19**, 365-377.

Pike, C. J. (1999). Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. *J. Neurochem.* **72**, 1552-1563.

Poli, G. and Parola, M. (1997). Oxidative damage and fibrogenesis. *Free Radicals Biol. Med.* **22**, 287-305.

Pugazhenthi, S., Nesterova, A., Jambal, P., Audesirk, G., Kern, M., Cabell, L., Eves, E., Rosner, M. R., Boxer, L. M., Reusch, J. E. (2003). Oxidative stress-mediated down-regulation of bcl-2 promoter in hippocampal neurons. *J. Neurochem.* **84**, 982-996.

Ratan, R. R., Murphy, T. H. and Baraban, J. M. (1994). Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.* **62**, 376-379.

Ruberg, M., France-Lanord, V., Brugg, B., Lambeng, N., Michel, P. P., Anglade, P., Hunot, S., Damier, P., Faucheux, B., Hirsch, E. and Agid, Y. (1997). Neuronal death caused by apoptosis in Parkinson disease. *Rev. Neurol.* **153**, 499-508.

Shoffner, J. M., Watts, R. L., Juncos, J. L., Torroni, A. and Wallace, D. C. (1991). Mitochondrial oxidative phosphorylation defects in Parkinson's disease. *Ann. Neurol.* **30**, 332-339.

Smith, M. A., Hirai, K., Hsiao, K., Pappolla, M. A., Harris, P. L., Siedlak, M., Tabaton, M. and Perry, G. (1998). Amyloid-beta deposition in Alzheimer transgenic mice is associated with oxidative stress. *J. Neurochem.* **70**, 2212-2215.

Whittemore, E. R., Loo, D. T. and Cotman, C. W. (1994). Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. *Neuroreport* **5**, 1485-1488.