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Induction of Antioxidative and Antiapoptotic Thioredoxin Supports Neuroprotective Hypothesis of Estrogen

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The original neuroprotective hypothesis of estrogen was based on the gender difference in brain response to the ischemia-reperfusion injury. Additional clinical reports also suggest that estrogen may improve cognition in patients with Alzheimer disease. 17β-Estradiol is the most potent endogenous ligand of estrogen, which protects against neurodegeneration in both cell and animal models. Estrogen-mediated neuroprotection is probably mediated by both receptor-dependent and -independent mechanisms. Binding of estrogen such as 17β-estradiol to estrogen receptors (ERs) activates the homodimers of ER-DNA and its binding to estrogen response elements in the promoter region of genes such as neuronal nitric oxide synthase (NOS1) for regulating gene expression in target brain cells. In addition to the induction of NOS1, estrogen increases the expression of antiapoptotic protein such as bcl-2. Furthermore, our recent observations provide new molecular biologic and pharmacologic evidence suggesting that physiologic concentrations of 17β-estradiol (<10 n*M*) activate ERs (ERβ > ERα) and upregulate a cyclic guanosine 5'-monophosphate (cGMP)-dependent thioredoxin (Trx) and MnSOD expression following the induction of NOS1 in human brain-derived SH-SY5Y cells. We thus proposed that the estrogen-mediated gene induction of Trx plays a pivotal role in the promotion of neuroprotection because Trx is a multifunctional antioxidative and antiapoptotic protein. For managing progressive neurodegeneration such as Alzheimer dementia, our estrogen proposal of the signaling pathway of cGMP-dependent protein kinase (PKG) in mediating estrogen-induced cytoprotective genes thus fosters research and development of the new estrogen ligands devoid of female hormonal side effects such as carcinogenesis.

Key Words: 17β-Estradiol; gene induction; SH-SY5Y cells; MnSOD; NOS1; thioredoxin.

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Brain Functions of Estrogen Receptor Subtypes

In addition to peripheral hormonal receptors, estrogen receptor α (ERα) and ERβ subtypes are widely distributed throughout the brain as nuclear binding sites *(1,2)*. In general, more brain neurons express ERβ than those containing ERα *(3)*. ERα sites are distributed mainly in the basal forebrain cholinergic neurons, while ERβ sites are predominantly expressed in the midbrain dopaminergic and serotonergic neurons. The development and plasticity of brain neurons can be modified by estrogen. Transfection of ERα leads to an increase in the number and length of neurites, whereas ERβ transfection results in neurite elongation *(4)*. Moreover, ERβ has a role in the survival of cortical, hippocampal, and nigral neurons *(3)*. However, ERα, but not ERβ, may mediate neuroprotection produced by physiologic concentrations of estrogen in ischemic animal models *(5,6)*. In addition to upregulation of Bcl-2, estrogen induces endothelial nitric oxide synthase (NOS3) in the cerebrovascular endothelial cells, thus mediating cytoprotective action *(7)*. Our unpublished reverse transcriptase polymerase chain reaction results indicate that human SH-SY5Y cells contain both ERα and ERβ subtypes. It is known that 17β-estradiol binds to both nuclear ERα and ERβ sites, which are sensitive to the receptor antagonist ICI 182,780 *(8)*. Physiologic concentrations of 17β-estradiol bind ICI 182,780-sensitive ER in SH-SY5Y cells, leading to protection against serum deprivation–induced oxidative stress *(9)*. This receptor-mediated neuroprotection is linked to the upregulation of cytoprotective genes such as *NOS1*, thioredoxin (*Trx*), and *MnSOD*, which is somewhat different from those acute antioxidative effects produced by micromolar concentrations of estrogen.

Studies using mice with knockout of either ERα or ERβ are necessary for investigating which subtype of ER mediates estrogen-induced brain functions including the multifaceted neuroprotective mechanisms. Studies on mice with either ERα or ERβ knockout suggest that both ER subtypes are needed not only for survival but also for learning. Spatial learning is drastically impaired while there is severe degeneration of neuronal cell bodies throughout the brain, especially in the substantia nigra of 2-yr-old mice with the knockout of ERβ *(3)*. These observations infer that ERβ more than ERα may play an important role in the pathogenesis of neurodegenerative diseases in the brain. Furthermore, there is

increased brain neuron sensitivity of ERβ null mice to excitatory toxicity caused by kainic acid.

The binding of estrogen ligands to ER promotes a high-affinity binding of ER-DNA-binding domain to the estrogen response elements for regulating target gene expressions *(10)*. Interestingly, ERα and ERβ may play different roles in gene regulation in the central nervous system *(11)*. ERβ rather than ERα mediates transcriptional activation of NOS1 by 17β-estradiol *(12)*, thereby activating cyclic guanosine 5'-monophosphate (cGMP)-dependent signaling pathway and promoting cGMP-dependent protein kinase (PKG)-mediated brain functions including the induction of the redox protein Trx *(9)*. ERβ gene, protein, and related signaling transduction pathways may be necessary not only for upregulating NOS1 but also for enhancing neuronal viability in the brain monoaminergic neurons via the induction of some of the beneficial cytoprotective genes such as *Trx*, *Bcl-2*, and *MnSOD*.

Multifaceted Neuroprotective Mechanisms of Estrogen

Numerous research groups are investigating why physiologic concentrations of estrogen improve cognition such as attention and short-term memory in some postmenopausal women with senile dementia of the Alzheimer type *(13–15)*. Estrogen-mediated neuroprotection is consistently observed in several oxidative stress models including serum deprivation *(16)*, toxic fragments of β-amyloid precursor protein *(17)*, quinolinic acid *(18)*, oxidized low-density lipoprotein *(19)*, human immunodeficiency virus type 1 (HIV-1) viral proteins (i.e., HIV-1 protease, gp-120, and Tat) *(20,21)*, and 1-methyl-4-phenylpyridinium (MPP^+) *(22)*. In fact, multifaceted neuroprotective mechanisms of estrogen have been proposed *(23–27)*. First, in addition to antiinflammation *(28)*, some studies have proposed that estrogen at micromolar concentrations may function as an atypical antioxidant owing to its unique phenolic ring A *(29,30)*. Second, it has been proposed that brain ERα and not ERβ mediates neuroprotection produced by physiologic concentrations of estrogen in ischemic animal models *(5,6)*. Third, estrogen promotes the expression of neurotrophic factors and antiapoptotic proteins, thus enhancing cell viability *(9,31–34)*. Fourth, estrogen modulates intracellular signaling cascades such as mitogen-activated protein kinase (MAPK), cyclic adenosine monophosphate–protein kinase A signal transduction pathway, and nontranscriptional signaling pathway, all of which may contribute to the promotion of neuroprotection *(28,35–37)*.

Besides these multifacted neuroprotective mechanisms of estrogen, it is also known that 17β-estradiol induces NOS1 and increases the synthesis of $^{\bullet}NO$ and cGMP in several cell models *(38–40)*. Recently, we have observed that transfection and/or expression of human NOS1 activates a cGMP-mediated signal transduction pathway and upregulates the expression of Trx, thereby preventing oxidative stress–induced apoptosis *(41–43)*. It is also known that NO (i.e., *S*-nitrosoglutathione) and 8-bromo-cGMP can individually protect cells and brain neurons against oxidative stress caused by serum deprivation, hydrogen peroxide, glutamate, toxic fragments of β-amyloid, and MPP^+ *(18,43–48)*. Moreover, our new observations suggest that the induction of NOS1 in human brain-derived SH-SY5Y cells may mediate the cGMP-dependent neuroprotection evoked not only by the preconditioning procedure *(41–44)* but also by physiologic concentrations of 17β-estradiol (<10 n*M*) *(9)*. This newly proposed Trx-dependent neuroprotective pathway of estrogen includes the induction of NOS1 following the activation of the ERβ rather than ERα, the cGMP-dependent protein kinase pathway, the phosphorylation of MAPK/Erk1/2, and the phosphoactivation of the transcription factor c-Myc to induce the Trx gene (Fig. 1). This new estrogen neuroprotective proposal is the first to bridge the gaps between estrogen-induced neuroprotection to the observed chain of events following the activation of ICI 182,780-sensitive ERs.

Pivotal Role of Trx Induction in Estrogen-Mediated Neuroprotection

The activation of ER has been suggested in mediating the complex mechanisms of neuroprotection, although by which mechanism estrogen induces the *Trx* gene is very intriguing. Trx is a multifunctional redox regulating protein, which has antioxidative and antiapoptotic actions implicated in cell proliferation, preconditioning adaptation, antioxidative defense, and apoptosis inhibition *(42,43,49,50)*. Analysis of the promoter region of Trx has revealed redox-sensitive regulatory elements. The present estrogen proposal was derived from our recent observation that Trx can be induced by preconditioning stress and plays a crucial role in gene expression such as Bcl-2 and MnSOD against oxidative stress–induced cell death *(44)*. The preconditioning induction of NOS1 activates the $^{\bullet}NO$-cGMP-PKG signaling pathway and increases the phosphorylation of MAPK/Erk1/2 and c-Myc, thereby inducing Trx for the promotion of antioxidation and neuroprotection *(43)*. With the fact that estrogen induces NOS1 following the activation of ERβ in mind *(12)*, we thus filled in the gaps in Fig. 1, proposing that 17β-estradiol may phosphoactivate Erk1/2 and c-Myc, thereby upregulating the *Trx* gene and protein following the induction of NOS1.

In addition to suppressing lipid peroxidation, membrane-permeable Trx-$(S)_2$ inhibits apoptosis by modulating the catalytic activity of the proapoptotic caspase-3 and apoptosis signal-regulating kinase 1 (ASK1) *(41–43,51,52)*. Estrogen-mediated neuroprotection can be prevented by the blockade of redox cycling of Trx from the inactive oxidized Trx-$(S)_2$ to the active reduced Trx-$(SH)_2$ by inhibitors of the selenium-containing Trx reductase *(9)*. Owing to its redox-reactive cysteinyl sites (-Trp-Cys^{32}-Gly-Pro-Cys^{35}-Lys-) and

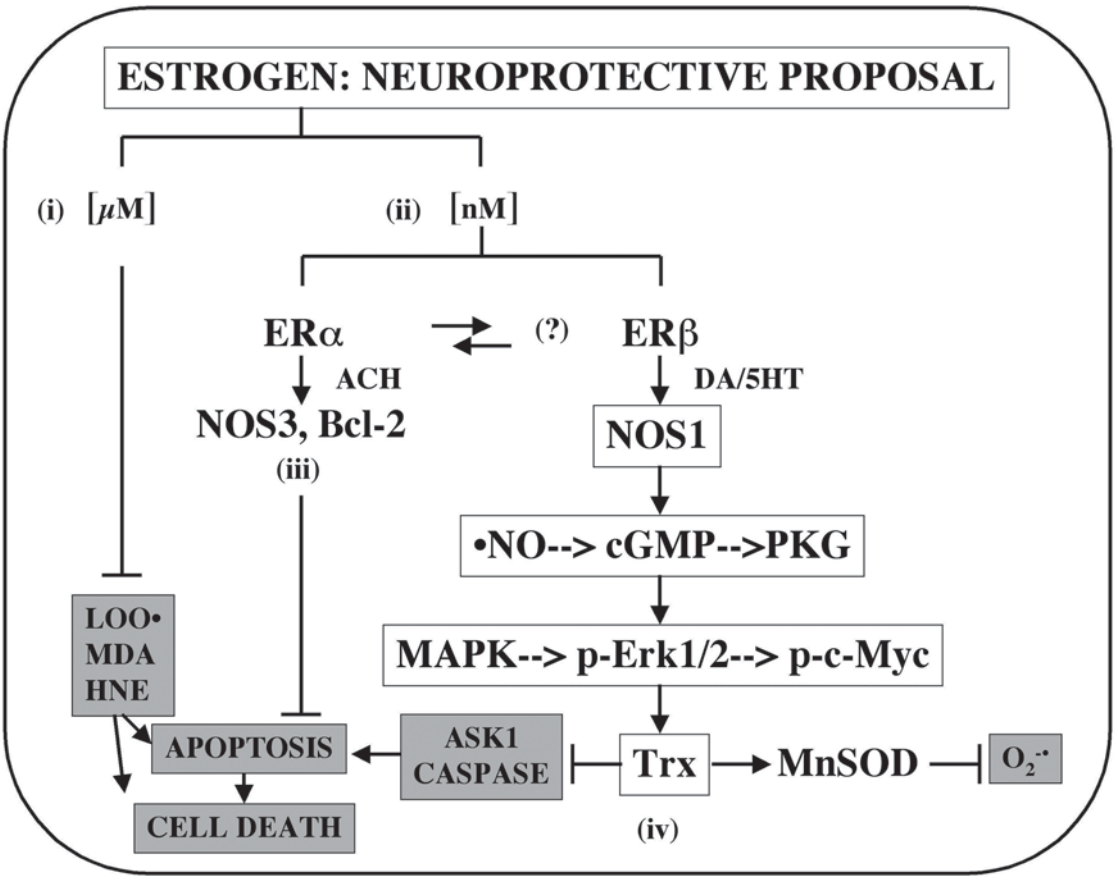

Fig. 1. Proposed cGMP-dependent gene induction of Trx in mediating neuroprotective mechanisms of estrogen. (i) At micromolar concentrations of estrogen, ligands such as 17α- and 17β-estradiol inhibit the chain reactions of lipid peroxidation such as the generation of lipid peroxyl radical (LOO•) and toxic lipid metabolites of malondialdehyde (MDA) and 4-hydroxyl-2-nonenal (HNE), which are known to cause necrosis and apoptosis. (ii) Physiologic concentrations (<10 n*M*) of 17β-estradiol bind with ERα and ERβ for promoting gene induction. (iii) ERα is distributed mainly in the forebrain cholinergic neurons (ACH). Through the activation of ERα, 17β-estradiol induces NOS3 (eNOS) in cerebrovasculature and also antiapoptotic protein Bcl-2 in the rodent brain, thereby protecting against ischemia-induced brain damage. (iv) ERβ is found in dopaminergic (DA) and serotonergic (5HT) neurons in the midbrain. It is known that ER (β >> α) upregulates the expression of NOS1 (nNOS) leading to the formation of nitric oxide (•NO), activation of the cGMP- and PKG-mediated signaling pathway, and phosphoactivation of Erk1/2 and c-Myc, which can subsequently induce cytoprotective proteins such as the redox protein Trx. Trx is known to inhibit apoptosis through the inactivation of ASK1, Apaf-1, and caspases. In addition to the inhibition of lipid peroxidation–induced cytotoxicity and caspase-induced apoptosis, Trx induces MnSOD to suppress the generation of reactive oxygen species (i.e., $O_2^{-\bullet}$, superoxide anion radical) in the mitochondria. This new neuroprotective mechanism of estrogen supports the neuroprotective hypothesis of estrogen derived from the gender difference in brain response to ischemic injury *(24,25)* since neuroprotection induced by physiologic concentrations of estrogen is mediated by both antioxidative and antiapoptotic actions produced by Trx and MnSOD *(9,60)*. Animals and cells overexpressing Trx or MnSOD are less vulnerable to oxidative stress caused by ischemia and MPP^+ neurotoxin. Therefore, the cGMP-induced cytoprotective genes such as *Trx* and *MnSOD* may play a pivotal role in enhancing cell viability to the promotion of neuroprotection following the treatment of human SH-SY5Y cells with physiologic concentrations of the most potent endogenous estrogen ligand, 17β-estradiol.

S-nitrosylation site (Cys^{69}), reduced Trx-$(SH)_2$ profoundly modulates the thiol-containing transcription factors (i.e., activation protein-1, nuclear factor κB), and the thiyl-containing enzymes such as caspases, Ref-1, ASK1, p53, and p21 *(49–56)*. Following the induction of Trx, estrogen also induces MnSOD but not HO-2 and brain-derived neurotrophic factor in human brain-derived SH-SY5Y cells *(9)*. It is known that Trx can upregulate MnSOD *(57)*; expression of mitochondrial MnSOD would augment the antioxidative effects of Trx. Therefore, the expression of Trx may play a pivotal role in mediating the neuroprotective mechanisms of estrogen because Trx is a multifunctional protein associated with gene induction for the promotion of cellular defense systems such as preconditioning adaptation, antioxidation, and apoptosis inhibition.

Conclusion

The present results infer that 17β-estradiol-enhanced expression of the multifunctional redox protein of Trx is the missing link between the NOS1 expression and the neuronal

survival following the treatment of brain neurons and other cells with physiologic concentrations of estrogen. These new observations are consistent with our prior proposal that preconditioning-induced hormesis or neuronal adaptation is mediated by Trx expression because it can be blocked by the antisense of Trx mRNA *(43)*. Moreover, mice overexpressing the redox protein Trx are less vulnerable in ischemia-induced brain injury *(49)*. Trx plays a critical regulatory role in nerve growth factor–mediated signaling transduction and outgrowth in PC12 cells *(58)*. Consistently, Trx also protects both SH-SY5Y and PC12 cells against severe oxidative stress and damage caused by the parkinsonism-producing neurotoxin MPP^+ *(41–43,46)*. This new proposal of a cGMP-dependent, Trx-mediated neuroprotective mechanism of estrogen supports the original estrogen neuroprotective hypothesis derived from the observations of a significant gender difference in the human brain response to ischemic injury, perhaps trauma and stroke as well *(24,25)*.

However, it is also known that the female hormonal actions of estrogen ($ER\alpha > ER\beta$) may promote ovarian and breast cancer, which certainly increase the risk to beneficial ratio in some postmenopausal women. Nevertheless, increasing reports suggest that some of the cyto- and neuroprotective effects of estrogen may be independent of female hormonal activity *(30,35,59)*. Our new estrogen proposal could stimulate further the emerging research field of pharmacogenomics using gene-inducing agents such as ER-modulating agents to awaken cytoprotective genes for the promotion of neuroprotection and to minimize the side effects of carcinogenesis *(42,60)*. This gene-awakening approach may have clinical implications for developing new neuroprotective agents, thereby slowing progressive neurodegeneration in brain disorders caused by cytotoxic reactive oxygen, nitrogen, and thiyl species such as Alzheimer dementia, Parkinson disease, trauma, stroke, and ischemia/reperfusion brain injury.

References

1. Belcher, S. M. (1999). *Brain Res. Dev. Brain Res.* **115,** 57–69.
2. McEwen, B., Akama, K., Alves, S., et al. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 7093–7100.
3. Wang, L., Andersson, S., Warner, M., and Gustafsson, J. A. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 2792–2796.
4. Patrone, C., Pollio, G., Vegeto, E., et al. (2000). *Endocrinology* **141,** 1839–1845.
5. Dubal, D. B., Zhu, H., Yu, J., et al. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 1952–1957.
6. Jover, T., Tanaka, H., Calderone, A., et al. (2002). *J. Neurosci.* **22,** 2115–2124.
7. Geary, G. G., McNeil, A. M., Ospina, J. A., Krause, D. N., Korach, K. S., and Duclles, S. P. (2001). *J. Appl. Physiol.* **91,** 2391–2399.
8. Howell, A., Osborne, C. K., Morris, C., and Wakeling, A. E. (2000). *Cancer* **89,** 817–825.
9. Lee, S. Y., Andoh, T., Murphy, D. L., and Chiueh, C. C. (2003). *FASEB J. Express.*10.1096/fj.02-0807fje.
10. Nishiyama, A., Matsui, M., Iwata, S., et al. (1999). *J. Biol. Chem.* **274,** 21645–21650.
11. Paech, K., Webb, P., Kuiper, G. G., et al. (1997). *Science* **277,** 1508–1510.
12. Nuedling, S., Karas, R. H., Mendelsohn, M. E., et al. (2001). *FEBS Lett.* **502,** 103–108.
13. Fillit, H., Weinreb, H., Cholst, I., et al. (1986). *Psychoneuroendocrinology* **11,** 337–345.
14. Shumaker, S. A., Reboussin, B. A., Espeland, M. A., et al. (1998). *Control Clin. Trials* **19,** 604–621.
15. Asthana, S., Baker, L. D., Craft, S., et al. (2001). *Neurology* **57,** 605–612.
16. Green, P. S., Bishop, J., and Simpkins, J. W. (1997). *J. Neurosci.* **17,** 511–515.
17. Kim, H., Bang, O. Y., Jung, M. W., et al. (2001). *Neurosci. Lett.* **302,** 58–62.
18. Kuroki, Y., Fukushima, K., Kanda, Y., Mizuno, K., and Watanabe, Y. (2001). *Eur. J. Neurosci.* **13,** 472–476.
19. Berco, M. and Bhavnani, B. R. (2001). *J. Soc. Gynecol. Invest.* **8,** 245–254.
20. Hawkins, V., Shen, Q., and Chiueh, C. C. (1999). *J. Biomed. Sci.* **6,** 433–438.
21. Howard, S. A., Brooke, S. M., and Sapolsky, R. M. (2001). *Exp. Neurol.* **168,** 385–391.
22. Sawada, H., Ibi, M., Kihara, T., et al. (2002). *Neuropharmacology* **42,** 1056–1064.
23. Wang, L., Andersson, S., Warner, M., and Gustafsson, J. A. (2002). *Sci. STKE* **138,** 1–4.
24. Hall, E. D., Pazara, K. E., and Linseman, K. L. (1991). *J. Cereb. Blood Flow Metab.* **11,** 292–298.
25. Roof, R. L. and Hall, E. D. (2000). *J. Neurotrauma* **17,** 367–388.
26. Garcia-Segura, L. M., Azcoitia, I., and DonCarlos, L. L. (2001). *Prog. Neurobiol.* **63,** 29–60.
27. Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W., and Bottner, M. (2001). *Endocrinology* **142,** 969–973.
28. Bruce-Keller, A. J., Keeling, J. L., Keller, J. N., Huang, F. F., Camondola, S., and Mattson, M. P. (2000). *Endocrinology* **141,** 3646–3656.
29. Rauhala, P. and Chiueh, C. C. (2000). *Ann. NY Acad. Sci.* **899,** 238–254.
30. Green, P. S., Yang, S. H., and Simpkins, J. W. (2000). *Novartis Found. Symp.* **230,** 202–213.
31. Ivanova, T., Kuppers, E., Engele, J., and Beyer, C. (2001). *J. Neurosci. Res.* **66,** 221–230.
32. Singleton, J. R., Randolph, A. E., and Feldman, E. L. (1996). *Cancer Res.* **56,** 4522–4529.
33. Dubal, D. B., Shughrue, P. J., Wilson, M. E., Merchenthaler, I., and Wise, P. M. (1999). *J. Neurosci.* **19,** 6385–6393.
34. Stoltzner, S. E., Berchtold, N. C., Cotman, C. W., and Pike, C. J. (2001). *Neuroreport* **12,** 2797–2800.
35. Sawada, H., Ibi, M., Kihara, T., et al. (2000). *FASEB J.* **14,** 1202–1214.
36. Paech, K., Webb, P., Kuiper, G. G., et al. (1997). *Science* **277,** 1508–1510.
37. Harms, C., Lautenschlager, M., Bergk, A., et al. (2001). *J. Neurosci.* **21,** 2600–2609.
38. Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 5212–5216.
39. Pelligrino, D. A., Santizo, R., Baughman, V. L., and Wang, Q. (1998). *Neuroreport* **9,** 3285–3291.
40. Garcia-Duran, M., de Frutos, T., Diaz-Recasens, J., et al. (1999). *Circ. Res.* **85,** 1020–1026.
41. Andoh, T., Chock, P. B., and Chiueh, C. C. (2002). *J. Biol. Chem.* **277,** 9655–9660.
42. Andoh, T., Chock, P. B., and Chiueh, C. C. (2002). *Ann. NY Acad. Sci.* **962,** 1–7.
43. Andoh, T., Chiueh, C. C., and Chock, P. B. (2003). *J. Biol. Chem.* **278,** 885–890.

44. Andoh, T., Lee, S. Y., and Chiueh, C. C. (2000). *FASEB J.* **14,** 2144–2146.
45. Estevez, A. G., Spear, N., Thompson, J. A., et al. (1998). *J. Neurosci.* **18,** 3708–3714.
46. Kim, Y. M., Chung, H. T., Kim, S. S., et al. (1999). *J. Neurosci.* **19,** 6740–6747.
47. Wirtz-Brugger, F. and Giovanni, A. (2000). *Neuroscience* **99,** 737–750.
48. Takuma, K., Phuagphong, P., Lee, E., Mori, K., Baba, A., and Matsuda, T. (2001). *J. Biol. Chem.* **276,** 48093–48099.
49. Takagi, Y., Mitsui, A., Nishiyama, A., et al. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 4131–4136.
50. Holmgren, A. (2000). *Antioxid. Redox Signal* **2,** 811–820.
51. Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J. M. (2000). *Mol. Cell Biol.* **20,** 2198–2208.
52. Hayashi, T., Ueno, Y., and Okamoto, T. (1993). *J. Biol. Chem.* **268,** 11380–11388.
53. Saitoh, M., Nishitoh, H., Fujii, M., et al. (1998). *EMBO J.* **17,** 2596–2606.
54. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 3633–3638.
55. Ueno, M., Masutani, H., Arai, R. J., et al. (1999). *J. Biol. Chem.* **274,** 35809–35815.
56. Haendeler, J., Hoffman, J., Tischler, V., Berk, B. C., Zeiher, A. M., and Dimmeler, S. (2002). *Nat. Cell Biol*. **4,** 743–749.
57. Das, K. C., Lewis-Molock, Y., and White, C. W. (1997). *Am. J. Respir. Cell Mol. Biol.* **17,** 713–726.
58. Bai, J., Nakamura, H., Kwon,Y.-W. et al. (2003). *J. Neurosci.* **23,** 503–509.
59. Behl, C., Skutella, T., Lezoualc'h, F., et al. (1997). *Mol. Pharmacol.* **51,** 535–541.
60. Chiueh, C. C. and Andoh, T. (2001). In: *Mapping the progress of Alzheimer's and Parkinson's disease.* (Mizuno, Y., Fisher, A., and Hanin, I. (eds.). Kluwer Academic/Plenum: New York.