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Cytochrome *c* Is a Potent Catalyst of Dichlorofluorescin Oxidation: Implications for the Role of Reactive Oxygen Species in Apoptosis

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The generation of reactive oxygen species has been suggested to occur at increased rates during apoptosis, but the validity and significance of this remain contentious. In several key studies levels of reactive oxygen species have been monitored using the intracellular probe dichlorofluorescin ($DCFH_2$), which undergoes oxidation to the fluorescent dichlorofluorescein (DCF). We report here that cytochrome *c*, which is released from mitochondria during cell death, is a potent catalyst of DCF formation. In a model system using xanthine oxidase to generate superoxide radicals, the rate of DCF formation was insensitive to changes in the rate of superoxide production over a 17-fold range, but extremely sensitive to nanomolar concentrations of cytochrome *c*. Thus we conclude that the DCF fluorescence observed in dying cells is a reflection of increased cytosolic cytochrome *c*. Moreover, we suggest that the suppression of DCF formation by the anti-apoptotic oncoprotein Bcl-2, which has been suggested to have antioxidant properties, can be explained on the basis of its prevention of mitochondrial cytochrome *c* release.

Key Words: **apoptosis; cytochrome *c;* reactive oxygen species; dichlorofluorescein.**

The mechanisms by which reactive oxygen species (ROS) can induce and modulate apoptosis have been the focus of considerable research over the past fifteen to twenty years (1–3). An important milestone was the demonstration by Kane *et al.* that neural cell death is accompanied by increased intracellular ROS generation, which is prevented by overexpression of the anti-apoptotic proto-oncogene *bcl-2* (4). This led to the view that increased ROS formation is necessary for the induction of apoptosis (programmed cell death) and that the Bcl-2 protein prevents cell death by acting as an antioxidant (5). However, this has been challenged by findings from three independent groups, which showed that programmed cell death (and its inhibition by Bcl-2) can occur under conditions of hypoxia, which should prevent ROS formation (6–8).

In nonphagocytic cells, the mitochondrial electron transport chain is the primary site of ROS generation. Indeed it has been known for two decades that approximately 2% of the oxygen consumed by mitochondria fails to undergo complete reduction to water and is instead released as the superoxide ($O_2^{\bullet -}$) radical (9–11), which yields hydrogen peroxide (H_2O_2) upon spontaneous disproportionation ($k_{obs} \approx 5.4 \times 10^5\ M^{-1}\ s^{-1}$ at pH 7.4) (12). In the landmark studies of Kane *et al.* (4), and indeed others (4, 5, 13), intracellular ROS levels were monitored using dichlorofluorescin ($DCFH_2$), a probe which undergoes oxidation to the fluorescent dichlorofluorescein (DCF) (14). The direct reaction between superoxide (or H_2O_2) and $DCFH_2$ is, however, very slow. Oxidation of the probe to the fluorescent DCF is much more efficient in the presence of a catalyst, such as the ferrous ion or a peroxidase (14, 15). Ferrous ions react with H_2O_2 to form the highly reactive hydroxyl radical ($^{\bullet}OH$) (16), whereas peroxidases, such as horseradish peroxidase or myeloperoxidase, are activated to oxidizing ferryl–heme intermediates by H_2O_2 (17). In view of the recent discovery that cytochrome *c* is released into the cytosol during apoptosis (18, 19), we have examined the possibility that this heme protein can also catalyze $DCFH_2$ oxidation. We show that cytochrome *c* is indeed a potent catalyst of DCF formation, suggesting that the DCF fluorescence observed in dying cells is a reflection of increased cytosolic cytochrome *c*. Moreover, we suggest that the suppression of DCF formation by Bcl-2 is due solely to its prevention of mitochondrial cytochrome *c* release.

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MATERIALS AND METHODS

Reagents. All incubations, which were carried out at 20°C, contained 50 mM Tris–HCl, 0.5 mM hypoxanthine and 0.1 mM diethylenetriaminepentaacetic acid, prepared by mixing solutions of the individual reagents, pre-adjusted to pH 7.4. The Tris–HCl and hypoxanthine stocks solutions, as well as the water used in all dilutions, were first treated with Chelex 100 resin to remove contaminating metal ions using the batch method (20). $DCFH_2$ was prepared from the diacetate by alkaline hydrolysis (14) and used at a final concentration of 10 μM. The spin trap DMPO was purified prior to use by vacuum distillation (Kugelrohr) and stored under N_2 at −80°C. All reagents were purchased from Sigma Chemical Co. (Dorset, United Kingdom).

Electron spin resonance. ESR spectra were recorded on a Bruker EMX spectrometer (X-band) with the following instrument settings: modulation frequency, 100 kHz; sweep width, 80 G; microwave power, 20 mW; modulation amplitude, 0.25 G; sweep time, 84 s; time constant, 41 ms; and 5×10^4 receiver gain. Four scans were accumulated and added. Hyperfine coupling constants were determined from spectral simulations performed using software available through the Internet (http://epr.niehs.nih.gov/) and described elsewhere (21). The coupling constants obtained were: a(N) = 14.14 G, $a(\beta$-H) = 11.31 G, $a(\gamma$-H) = 1.25 G for the DMPO/$^{\bullet}$OOH adduct and a(N) = 15.06 G, $a(\beta$-H) = 14.59 G for the DMPO/$^{\bullet}$OH adduct, which are in good agreement with literature values (22).

Fluorescence and visible spectrophotometry. $DCFH_2$ (10 μM) oxidation to DCF was monitored by fluorescence spectroscopy using a Perkin–Elmer LS 50 B instrument with excitation at 502 nm and emission at 540 nm. To measure superoxide production, the reduction of acetylated cytochrome *c* (25 μM) was monitored at 550 nm (23).

RESULTS

To mimic the production of superoxide radicals by respiring mitochondria in a well-defined system, we used xanthine oxidase (XO) with hypoxanthine as substrate (24). The generation of $O_2^{\bullet -}$ was confirmed using electron spin resonance (ESR) spectroscopy in conjunction with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (25). Incubation of 2×10^{-2} units ml^{-1} XO with DMPO resulted in the detection of an ESR spectrum consisting of a prominent signal from the DMPO superoxide adduct (DMPO/$^{\bullet}$OOH), confirming generation of the radical (Fig. 1a). The spectrum also contained a signal from the $^{\bullet}$OH radical adduct (DMPO/$^{\bullet}$OH). Inclusion of 2 M dimethyl sulfoxide (DMSO) in the system had no effect on the intensity of the DMPO/$^{\bullet}$OOH signal, but the DMPO/$^{\bullet}$OH signal was replaced by the DMPO methyl radical adduct, DMPO/$^{\bullet}CH_3$ (data not shown). Since $^{\bullet}$OH radicals are converted to $^{\bullet}CH_3$ radicals upon scavenging by DMSO ($k = 7 \times 10^9\ M^{-1}\ s^{-1}$) (26), this observation confirms the generation of free $^{\bullet}$OH radicals. Hydroxyl radical generation was insensitive to the presence of catalase (not shown), indicating that the radical was not generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Instead, it is concluded that $^{\bullet}$OH radicals are generated in this system as a spin-trapping artifact via

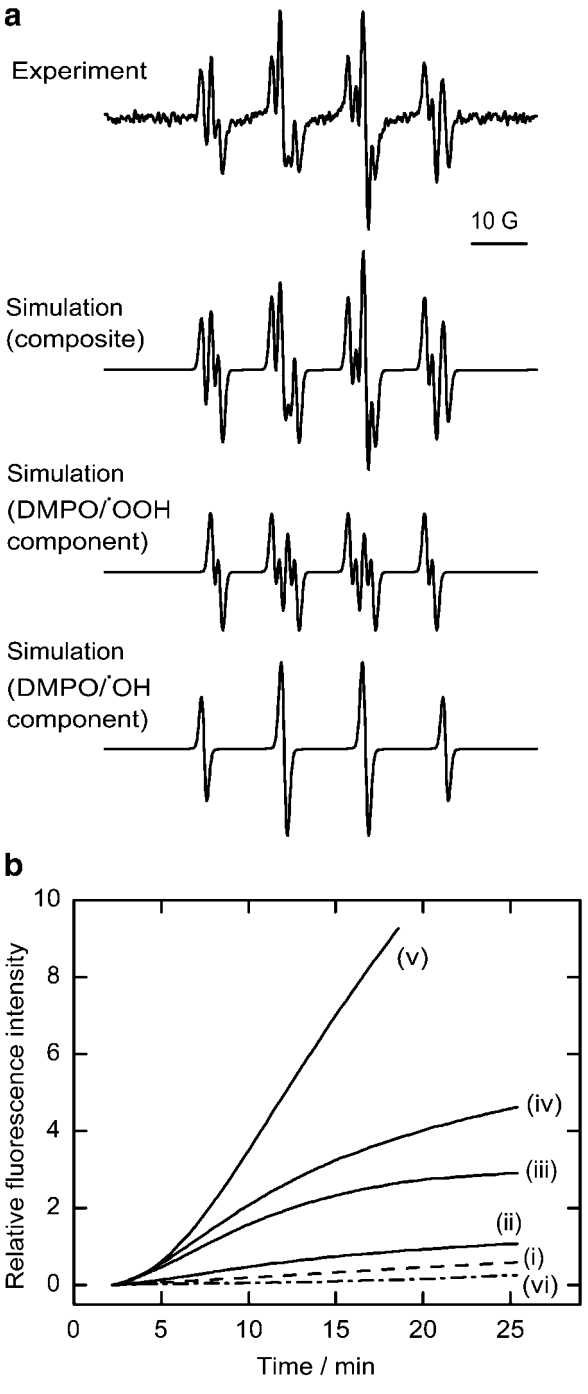

FIG. 1. Superoxide generation and its cytochrome *c*-catalyzed oxidation of $DCFH_2$. (a) Superoxide radicals were detected by ESR spectroscopy after 5 min in an incubation containing 2×10^{-2} units ml^{-1} XO and the spin trap DMPO. The simulated composite spectrum consists of signals from DMPO-trapped superoxide (DMPO/$^{\bullet}$OOH) and hydroxyl (DMPO/$^{\bullet}$OH) radicals in the relative ratio 0.72:0.28. (b) $DCFH_2$ (10 μM) oxidation to DCF was monitored by fluorescence spectroscopy in reaction mixtures containing 4×10^{-3} units ml^{-1} XO, alone (i) and with 1 (ii), 5 (iii), 10 (iv), or 25 nM (v) cytochrome *c*. Trace vi is from an incubation containing 25 nM cytochrome *c* but no XO.

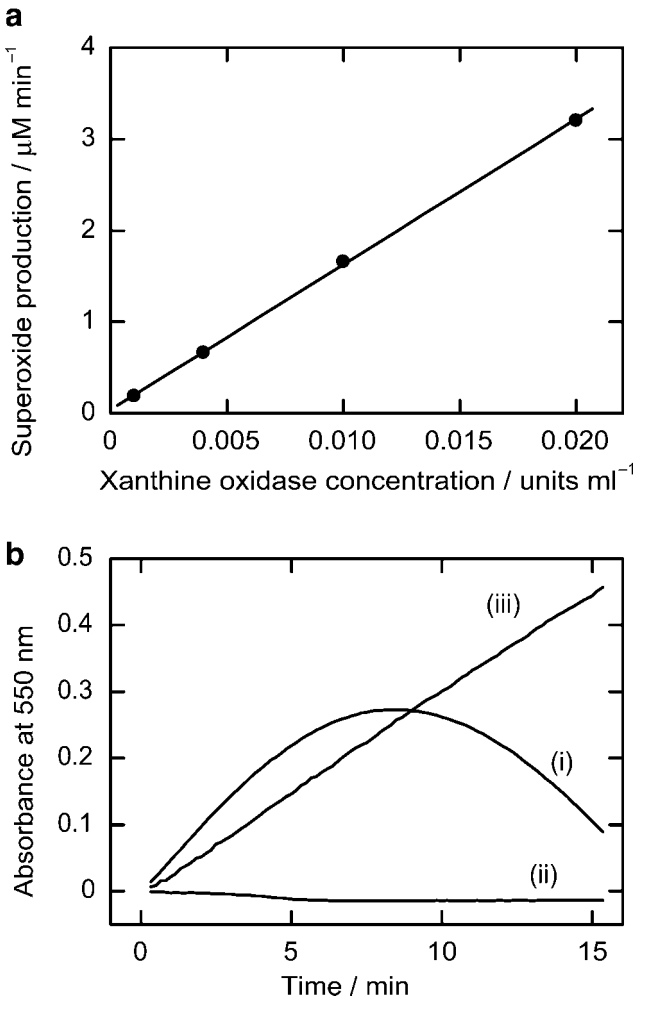

FIG. 2. Superoxide-dependent reduction of acetylated ferricytochrome *c*. (a) Rates of superoxide radical generation in the presence of XO were determined from initial rates of acetylated ferricytochrome *c* (25 μM) reduction, monitored at 550 nm. In all cases, reduction of the cytochrome was inhibited totally by SOD. Values determined in triplicate showed less than 1% variation. (b) Acetylated ferricytochrome *c* reduction in the presence 2×10^{-2} units ml^{-1} XO, alone (i) and with the addition of either 187 units ml^{-1} SOD (ii) or 214 units ml^{-1} catalase (iii).

the decomposition of DMPO/$^{\bullet}$OOH, as reported by Lloyd and Mason *et al.* (27), confirming their conclusion that only $O_2^{\bullet-}$ and H_2O_2 are generated by XO.

Incubation of $DCFH_2$ with 4×10^{-3} units ml^{-1} XO failed to induce significant DCF generation (Fig. 1b). At this concentration of XO, the rate of $O_2^{\bullet-}$ production was 0.66 μM min^{-1} (Fig. 2a), as determined from the rate of superoxide dismutase (SOD)-sensitive acetylated ferricytochrome *c* reduction (23). The inclusion of nanomolar concentrations of cytochrome *c* in the reaction system resulted in extensive, concentration-dependent $DCFH_2$ oxidation. The cytochrome was unable to oxidize the probe in the absence of XO (Fig. 1b).

Mitochondria isolated from apoptotic cells have been shown to produce increased amounts of $O_2^{\bullet-}$, which is attributed to their loss of cytochrome *c* (3). To vary the rate of $O_2^{\bullet-}$ production in our well-defined model system, varying concentrations of XO were employed. As shown in Fig. 2a, the rate of $O_2^{\bullet-}$ generation increased linearly with XO concentration. After prolonged incubation, especially at the higher XO concentrations, the accumulated ferrocytochrome *c* was seen to undergo oxidation back to the ferric form, which was prevented by catalase (Fig. 2b). This reflects the oxidation of ferrocytochrome *c* by H_2O_2, which is generated by the spontaneous disproportionation of superoxide and removed by catalase. No increase in absorption at 550 nm was seen in incubations containing SOD (Fig. 2b), which catalyses the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2 ($k > 10^9$ M^{-1} s^{-1} at pH 7.0) (12, 28), thereby preventing reduction of the cytochrome by superoxide. The small *decrease* in absorbance seen in the presence of SOD is attributed to the oxidation of contaminating acetylated ferrocytochrome *c* by H_2O_2 (Fig. 2b). Complementary ESR spin-trapping measurements provided direct, spectroscopic evidence for superoxide production at each XO concentration (not shown). The oxidation of $DCFH_2$ was then monitored at each concentration of XO, in both the presence and absence of 10 nM cytochrome *c*. Although low levels of $DCFH_2$ oxidation did occur in the absence of cytochrome *c* at the higher XO concentrations used, in all cases the cytochrome markedly enhanced formation of the fluorescent DCF (Figs. 3a–3d). The rate of $DCFH_2$ oxidation was not always proportional to the rate of $O_2^{\bullet-}$ generation, which may reflect the operation of complex secondary reactions involving the semiquinone form of the probe (15). The promotion of $DCFH_2$ oxidation by cytochrome *c* was striking in that more DCF was produced at the lowest concentration of XO used (1×10^{-3} units ml^{-1}, producing 0.18 μM $O_2^{\bullet-}$ min^{-1}) in the presence of cytochrome *c* than at the highest concentration of XO (2×10^{-2} units ml^{-1}, producing 3.20 μM $O_2^{\bullet-}$ min^{-1}) in the absence of the cytochrome (Figs. 3a and 3d).

DISCUSSION

The above findings may have serious implications regarding the role of ROS in apoptosis and the apparent antioxidant properties of Bcl-2. Like XO, mitochondria generate $O_2^{\bullet-}$ and H_2O_2, yet these species react only slowly with $DCFH_2$ when compared with the powerful oxidants formed following their interactions with ferrous ions, peroxidases and cytochrome *c*. Therefore $DCFH_2$ cannot be used to compare rates of $O_2^{\bullet-}$ and H_2O_2 production between systems containing different levels of any of these catalysts, such as apoptotic and non-apoptotic cells (13). We have shown, for example, that the presence of cytochrome *c* can result a substan-

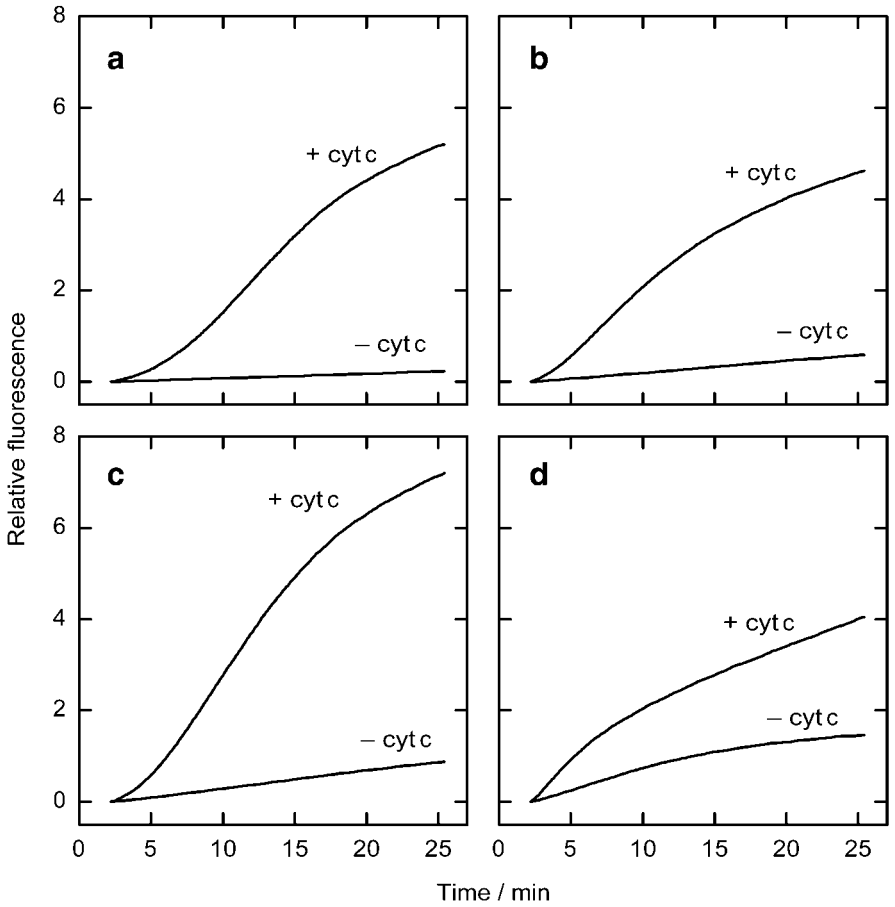

FIG. 3. Effect of cytochrome *c* (cyt *c*) on DCF formation in the presence of superoxide radicals generated at various rates. $DCFH_2$ (10 μM) oxidation to DCF was monitored by fluorescence spectroscopy (excitation 502 nm; emission 540 nm) in the presence of XO at concentrations of 1×10^{-3} (a), 4×10^{-3} (b), 1×10^{-2} (c) 2×10^{-2} units ml^{-1} (d) (yielding superoxide radicals at the rates shown in Fig. 2b), in both the absence and presence of 10 nM cytochrome *c*.

tial increase in the rate of $DCFH_2$ oxidation, despite a lowered (17-fold) rate of $O_2^{\cdot-}$ and H_2O_2 production. Therefore, in the context of the apoptotic cell, DCF fluorescence must be considered to be a measure of cytosolic cytochrome *c* rather than $O_2^{\cdot-}$ and H_2O_2.

We propose that the increased DCF fluorescence observed during neural cell death reported in the key paper of Kane *et al.* (4), which preceded the finding that cytochrome *c* is released from mitochondria during cell death (18, 19), is a reflection of the catalysis of $DCFH_2$ oxidation by cytochrome *c*. The demonstration by these authors that *bcl-2* overexpression results in the suppression of $DCFH_2$ oxidation can now be explained solely on the basis that the oncoprotein prevents the mitochondrial release of cytochrome *c* (18, 19). As shown here, the presence of cytochrome *c*, at concentrations well within those necessary to trigger the final stages of apoptosis (29–31), is the most important determinant of the rate of $DCFH_2$ oxidation, overriding any effects of a change in the rate of $O_2^{\cdot-}$ and H_2O_2 production. On the basis of the observations of Kane *et al.* (4), one can conclude that Bcl-2 acts as an antioxidant only insofar as it prevents cytochrome *c* becoming available as a catalyst for the conversion of background $O_2^{\cdot-}$ and H_2O_2 to a reactive oxidant (either $^{\cdot}OH$ or an activated peroxidase-type species).

More recently, Cai and Jones, using the SOD-sensitive reduction of acetylated ferricytochrome *c* to measure $O_2^{\cdot-}$, showed that mitochondria isolated from apoptotic cells produce the radical at higher rates than control cells (3). This is believed to result from a switch from the normal 4-electron reduction of O_2 to H_2O by cytochrome *c* oxidase to its 1-electron reduction to $O_2^{\cdot-}$ at a site upstream of cytochrome *c*, which is a direct consequence of the interrupted electron flow caused by loss of the cytochrome. Cai and Jones also found that *bcl-2* overexpression blocks the SOD-sensitive reduction of acetylated ferricytochrome *c* (3), which we suggest is a simple consequence of the prevention of cytochrome *c* release by Bcl-2 and not due to any intrinsic antioxidant property of the oncoprotein. Thus, together with reports on the ability of Bcl-2 to inhibit apoptosis

under hypoxic conditions (6–8), our findings bring into question the significance of the observations that have led to the hypothesis that Bcl-2 prevents cell death by acting as an antioxidant.

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REFERENCES

1. Jacobsen, M. D. (1996) *Trends Biochem. Sci.* **21,** 83–86.
2. Hampton, M. B., and Orrenius, S. (1998) *BioFactors* **8,** 1–5.
3. Cai, J., and Jones, D. P. (1998) *J. Biol. Chem.* **273,** 11401–11404.
4. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Örd, T., and Bredesen, D. E. (1993) *Science* **262,** 1274–1277.
5. Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **75,** 241–251.
6. Muschel, R. J., Bernhard, E. J., Garza, L., McKenna, W. G., and Kock, C. J. (1995) *Cancer Res.* **55,** 995–998.
7. Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995) *Nature* **374,** 811–813.
8. Jacobsen, M. D., and Raff, M. C. (1995) *Nature* **374,** 814–816.
9. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev.* **59,** 527–605.
10. Turrens, J. F., and Boveris, A. (1980) *Biochem. J.* **191,** 421–427.
11. Turrens, J. F. (1997) *Bioscience Reports* **17,** 3–8.
12. Bielski, B. H. L., Cabelli, D. E., and Arudi, R. L. (1985) *J. Phys. Chem. Ref. Data* **14,** 1041–1100.
13. Esposti, M. D., Hatzinisiriou, I., McLennan, H., and Ralph, S. (1999) *J. Biol. Chem.* **274,** 29831–29837.
14. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.* **5,** 227–231.
15. Rota, C., Fann, Y. C., and Mason, R. P. (1999) *J. Biol. Chem.* **274,** 28161–28168.
16. Burkitt, M. J. (1993) *Free Radical Res. Commun.* **18,** 43–57.
17. Dunford, H. B. (1995) *Xenobiotica* **25,** 725–733.
18. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132.
19. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136.
20. Buettner, G. R. (1988) *J. Biochem. Biophys. Methods* **16,** 27–40.
21. Duling, D. (1994) *J. Magn. Res. B* **104,** 105–110.
22. Buettner, G. R. (1987) *Free Radical Biol. Med.* **3,** 259–303.
23. Azzi, A., Montecucco, C., and Richter, C. (1975) *Biochem. Biophys. Res. Commun.* **65,** 597–603.
24. Fridovich, I. (1970) *J. Biol. Chem.* **245,** 4053–4057.
25. Mason, R. P., Hanna, P. M., Burkitt, M. J., and Kadiiska, M. B. (1994) *Environ. Health Perspect.* **102**(Suppl. 10), 33–36.
26. Veltwisch, D., Janata, A., and Asmus, K.-D. (1980) *J. Chem. Soc. Perkin Trans.* **2,** 146–153.
27. Lloyd, R. V., and Mason, R. P. (1990) *J. Biol. Chem.* **265,** 16733–16736.
28. Klug, D., Rabani, J., and Fridovich, I. (1972) *J. Biol. Chem.* **247,** 4839–4842.
29. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489.
30. Zhivotosky, B., Orrenius, S., Brustugun, O. T., and Døskeland, S. O. (1998) *Nature* **391,** 449–450.
31. Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., and Orrenius, S. (1999) *EMBO J.* **18,** 2040–2048.