Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells

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Abstract Mitochondrial depolarisation has been reported to enhance the generation of superoxide anion ($O_2^{\bullet-}$) in a number of cell preparations while an inhibition has been observed with isolated mitochondria. Cerebellar granule cells equilibrated with > 1 μM hydroethidine (dihydroethidium) which is oxidised to the fluorescent ethidium cation by $O_2^{\bullet-}$ showed a large increase in fluorescence on protonophore addition. However, controls showed the fluorescent enhancement to be a consequence of release of unbound preformed ethidium from the mitochondrial matrix within the cell with resultant fluorescent enhancement. This ambiguity was removed by the use of low (1 μM) concentrations of dye in which case generated ethidium remained bound within the mitochondria. Under these conditions antimycin A, but not protonophore addition, produced an increase in fluorescence. It is concluded that excess ethidium acts as an indicator of mitochondrial membrane potential obscuring the monitoring of $O_2^{\bullet-}$ and that certain experiments employing this indicator in cells may require re-evaluation.

Key words: Mitochondrion; Reactive oxygen species; Neuron; Mitochondrial membrane potential; Superoxide

1. Introduction

A major source of superoxide ($O_2^{\bullet-}$) in cells is complex III of the mitochondrial respiratory chain where transient quinone radicals are intermediates in the chemiosmotic Q-cycle (reviewed in [1]). The large pool of free ubiquinol/ubiquinone (UQH_2/UQ) which exists in the membrane shuttles electrons to complex III, where oxidation of the quinol takes place in two 1 e^- stages: the first electron is transferred to the Rieske protein with the release of two protons and generation of the semiquinone $UQ^{\bullet-}$; the second electron is transferred to the cytochrome b_L haem close to the cytoplasmic face of the membrane. Reoxidation of b_L involves transfer of the electron across the membrane to the second haem (b_H) close to the matrix face. This electron transfer is opposed by the membrane potential ($\Delta\psi_m$) and the steady-state concentration and half-life of $UQ^{\bullet-}$ increases in respiratory state 4 [2,3], enhancing a 1 e^- non-enzymatic transfer to molecular oxygen with the generation of $O_2^{\bullet-}$. Consistent with this, direct determinations with isolated mitochondria of $O_2^{\bullet-}$ [1,4] and the superoxide dismutase product H_2O_2 [5] show a steep correlation between $\Delta\psi_m$ and the rate of $O_2^{\bullet-}$ generation, such that the latter is abolished by protonophores or even the modest decrease in $\Delta\psi_m$ during ATP synthesis (reviewed in [1]).

There is considerable current interest in the role of mitochondrial free radicals in the initiation of cellular necrosis and apoptosis in the brain and immune system (reviewed in [6–8]). However, there is ambiguity in studies with isolated cells as to whether protonophores inhibit [9] or, in contradiction to isolated mitochondrial studies, enhance [10–15] the production of reactive oxygen species. Many of these latter studies have assayed $O_2^{\bullet-}$ production by monitoring the oxidation of hydroethidine (HET, also called dihydroethidium) to the fluorescent ethidium [11–15] and in this paper we attempt to resolve the nature of this discrepancy by comparing the behaviour of this indicator system in both intact cells (cultured cerebellar granule cells) and isolated rat liver mitochondria. Our conclusion is that while HET is an authentic detection system for $O_2^{\bullet-}$, the mitochondrial membrane-potential-dependent distribution of excess, unbound ethidium cation across the mitochondrial inner membrane critically affects its fluorescent yield. As first reported by Rottenberg [16], ethidium distributes across the mitochondrial membrane in response to the membrane potential, $\Delta\psi_m$: thus intracellular mitochondrial depolarisation results in a rapid efflux from the matrix, allowing the cation to bind to nuclear DNA with an extensive fluorescent enhancement. It is concluded that the enhanced fluorescence, seen when HET-loaded cells are depolarised, reflects an enhanced fluorescent yield of preformed ethidium rather than an increase in $O_2^{\bullet-}$ generation. This ambiguity is avoided by the use of low concentrations of HET, in which case an enhanced production of $O_2^{\bullet-}$ by antimycin A, but not by FCCP, can be observed.

2. Materials and methods

Cerebellar granule cells were prepared essentially as described by Courtney et al. [17], from 7-day-old Wistar rats, and plated on poly-D-coated coverslips at a density of 280 000 per coverslip and cultured in minimal essential medium supplemented with foetal calf serum (10% v/v), 25 mM KCl, 30 mM glucose, 2 mM glutamine, 50 U/ml of penicillin and 50 μg/ml of streptomycin. At 24 h in vitro 10 μM cytosine arabinoside was added. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air for 7–9 days. Coverslip-mounted cells were incubated with 5 μM hydroethidine or 5 μM ethidium (Figs. 1 and 2) in a medium composed of: 153 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 1.2 mM $NaSO_4$, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$ and 15 mM glucose for 10 min in the dark at 37°C and rinsed before being visualised at 485 nm excitation and > 515 nm emission with an inverted Nikon Diaphot epifluorescence microscope equipped with a Life Science Resources MiraCal imaging system. In a separate experiment (Fig. 4) 1 μM or 10 μM HET was present throughout the experiment. Ethidium fluorescence was relatively photostable under normal illumination conditions and auto-oxidation in granule cells was negligible for at least 30 min.

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Abbreviations: HET, hydroethidine; $\Delta\psi_m$, mitochondrial membrane potential

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3. Results

Depolarisation of mitochondria in situ within cultured cerebellar granule cells by the combination of rotenone to inhibit mitochondrial complex I and oligomycin to inhibit the ATP synthase [18] has been shown to confer a high degree of protection against glutamate excitotoxicity [19]. $O_2^{\bullet-}$ is believed to play a central role in this degenerative process [6–8] and experiments with isolated mitochondria (reviewed in [1]) would predict that the depolarisation would inhibit the mitochondrial production of $O_2^{\bullet-}$. The oxidation of HET to ethidium has been widely employed to monitor $O_2^{\bullet-}$ generation within cells and to support a paradoxical enhancement of $O_2^{\bullet-}$ generation by factors which depolarise the mitochondrion [11–15]. This counter-intuitive enhancement of fluorescence was found in the present preparation (Fig. 1A); however, a number of features suggest that the fluorescence is primarily monitoring $\Delta\psi_m$ rather than $O_2^{\bullet-}$: thus oligomycin, which hyperpolarises in situ mitochondria [20], decreased the fluorescence; rotenone plus oligomycin, which causes total depolarisation [18,20], caused a large enhancement, while rotenone alone, which allows $\Delta\psi$m to be supported by hydrolysis of cytoplasmic ATP and only slightly depolarises the mitochondria [18,20], caused a slight enhancement.

Since these findings suggest that a generally accepted correlation may require re-evaluation, a detailed study was performed. When cultured cerebellar granule cells were incubated with HET for 10 min and viewed by digital imaging, morphologically intact cells showed a low fluorescence which increased only slowly with time (Fig. 1A). As reported by Bindokas et al. [13] this fluorescence was punctate (Fig. 2A) and localised to mitochondria which in granule cells occupy an annulus around the large central nucleus. Addition of the protonophore FCCP to cells which have been incubated with HET produced a rapid enhancement of fluorescence (Fig. 1A) and at the same time the distribution of the fluorescence changed from a punctate appearance to a diffuse cytoplasmic/nuclear staining (Fig. 2B).

The enhanced signal has been observed in cultured hippocampal neurones [13] and lymphocytes [11] and has been interpreted as an increased rate of superoxide generation following mitochondrial depolarisation. However, the cationic ethidium distributes freely across mitochondrial inner membranes in response to the membrane potential [16]; thus an alternative explanation could be that the fluorescent yield of the ethidium produced by the oxidation of HET and accumulated within the mitochondrion could be enhanced on release from the mitochondrial matrix. In order to assess the contribution such a redistribution would make to the signal, a parallel experiment was performed in which cells were incubated in the presence of 5 μM ethidium (Fig. 1B). Sufficient ethidium accumulated to produce a punctate fluorescence in morphologically healthy cells (Fig. 2E); however, addition of FCCP caused a rapid enhancement (Fig. 1A) and diffusion (Fig. 2F) of the fluorescence which was closely identical to that observed in the HET experiment. Thus most or all of the protonophore-induced fluorescence increase in the HET experiment can be ascribed to redistribution of pre-existing ethidium rather than enhanced $O_2^{\bullet-}$ generation.

HET detects $O_2^{\bullet-}$ in a cell-free assay (Fig. 3A): the detection of the free radical generated by xanthine/xanthine oxidase is prevented by addition of superoxide dismutase, indicating that

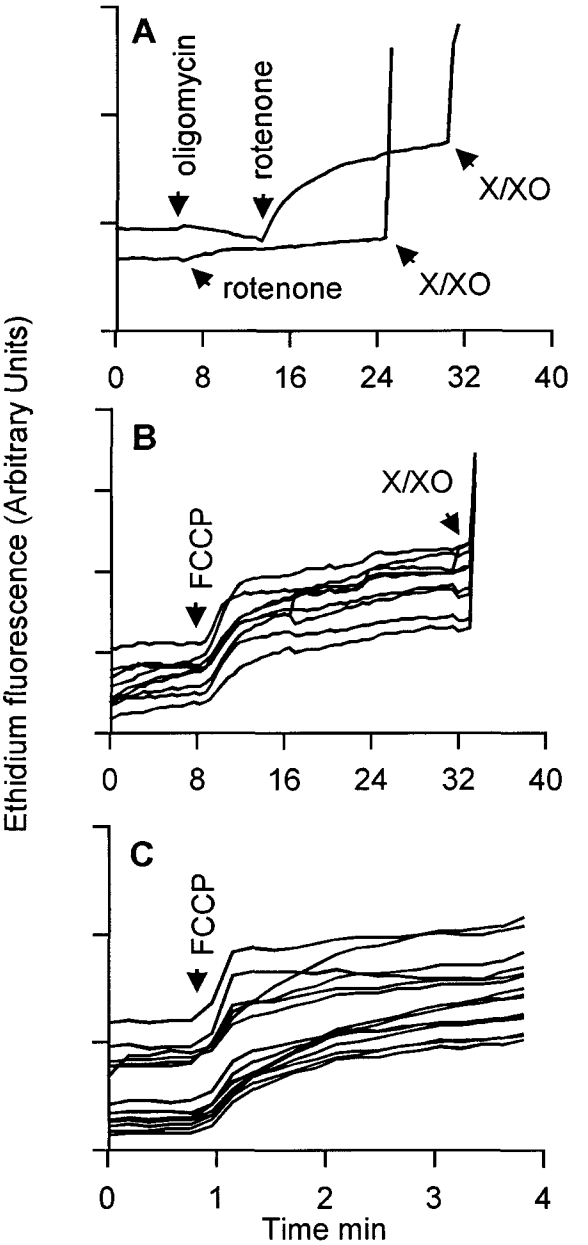

Fig. 1. A, B: Single cell fluorescence of a culture of cerebellar granule cells incubated in the presence of 5 μM HET for 20 min. In A, each trace represents the mean fluorescence within the somata of 10 individual cells. In B individual cell responses are shown. Additions were made where indicated of 10 μM oligomycin, 5 μM rotenone, 100 μM xanthine plus 10 mU xanthine oxidase (X/XO), or 2 μM FCCP. C: Cells were incubated in the presence of 5 μM ethidium. Note that the fluorescence increase parallels that shown in B. Results for each experiment are representative of 3–4 independent experiments from different cultures.

the assay does not detect generated H_2O_2, while the assay only slowly responds to high concentrations of added H_2O_2.

Ethidium binds avidly to nucleic acids and Fig. 3B shows the large fluorescent enhancement produced by the addition of DNA to a cell-free assay. For HET to accurately monitor $O_2^{\bullet-}$ under conditions where $\Delta\psi_m$ may vary it is therefore necessary to eliminate changes in fluorescent yield due to redistribution of excess unbound ethidium. This can be achieved by decreasing the concentration of HET such that ethidium is not gen-

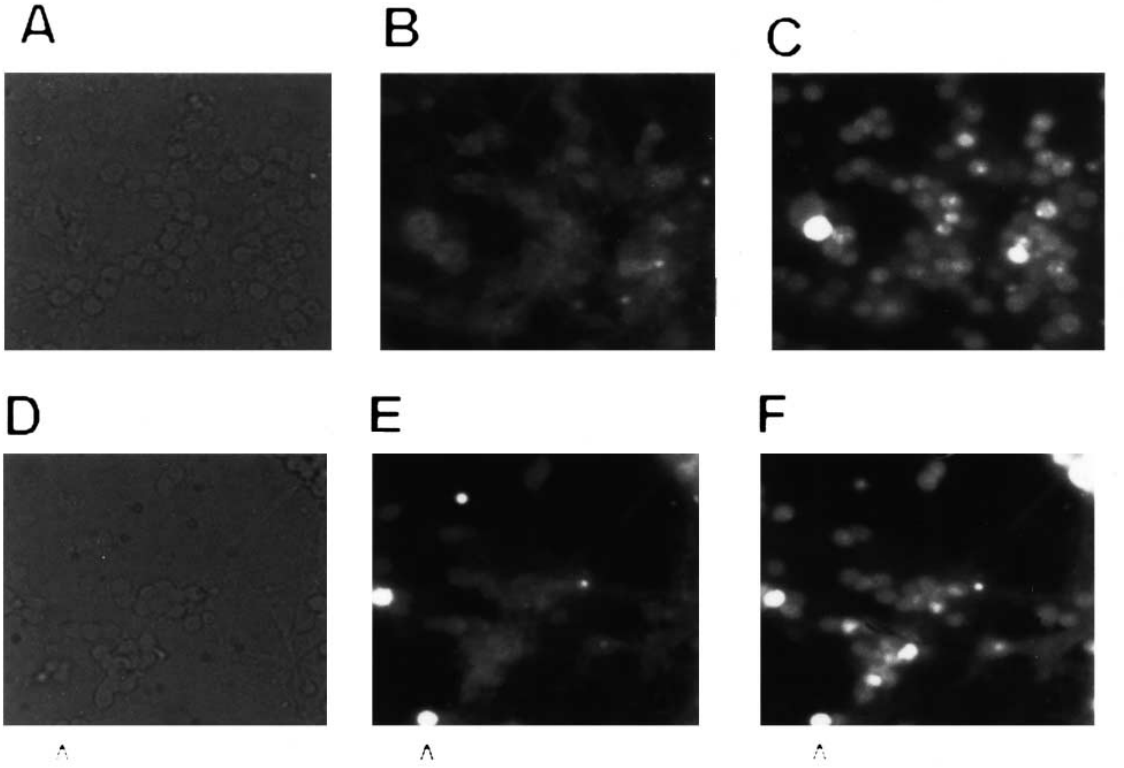

Fig. 2. Fluorescence of cerebellar granule cells incubated with 5 μM HET or ethidium in response to protonophore-induced depolarization of mitochondria. A: Bright-field digital image of cells prior to loading with 5 μM HET. B: Digital fluorescent image of the same field of cells after loading with 5 μM HET and incubation for 20 min. C: The same field 2 min after addition of 2 μM FCCP. Note the punctate appearance of the fluorescence in B and the enhanced, diffuse fluorescence after addition of the protonophore. D, E, F: Respective bright-field and fluorescent images of cells loaded with 5 μM ethidium and imaged before and 2 min after addition of 2 μM FCCP. Note that a morphologically damaged cell (arrowed) displays a high fluorescence both before and after FCCP addition.

erated beyond the binding capacity of mitochondrial nucleic acids. Fig. 4 shows that the protonophore-mediated enhancement in fluorescence is only observed in cells equilibrated with high concentrations of HET. The ability to monitor authentic superoxide generation after equilibration with low concentrations of HET under these conditions is confirmed by the enhanced rate of fluorescence increase on addition of antimycin A, consistent with results obtained with isolated mitochondria (reviewed in [1]).

4. Discussion

These results help to resolve some of the apparent contradictions between the effect of $\Delta\psi_m$ on $O_2^{\bullet-}$ generation by isolated mitochondria and by mitochondria in situ within the functioning cells, and emphasise the complexities inherent in assaying mitochondrial $O_2^{\bullet-}$ generation in intact cells. None of the currently employed fluorescent probes of reactive oxygen is without complications: dichlorodihydrofluorescein deriva-

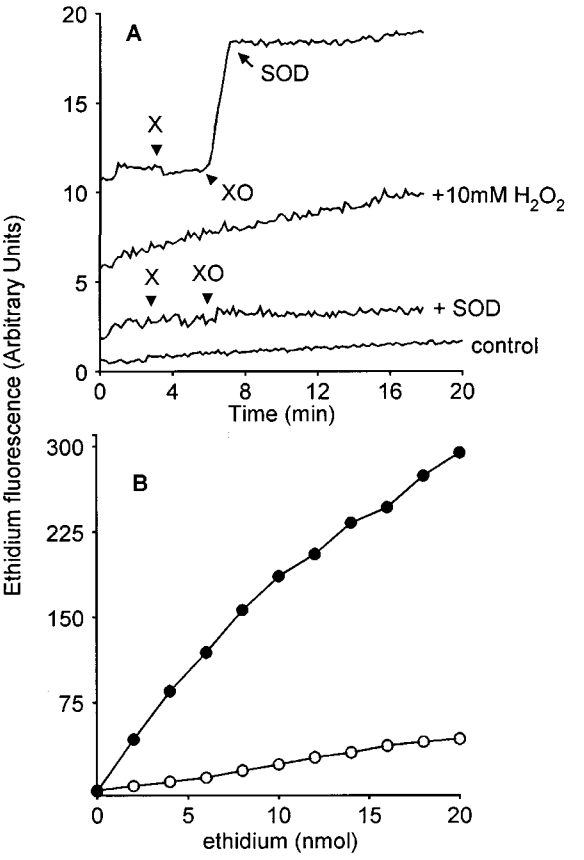

Fig. 3. A: Oxidation of HET by $O_2^{\bullet-}$ generated by xanthine/xanthine oxidase (X/XO) in a cell-free assay: note that HET is oxidized by X/XO but that this is abolished in the presence of 50 U/ml superoxide dismutase (SOD) indicating that the dye is not oxidized by generated H_2O_2. However, high concentrations of added H_2O_2 cause a slow increase [14]. Traces are displaced vertically for clarity. B: Fluorescent enhancement of ethidium standards in the presence of 100 μg/ml DNA.

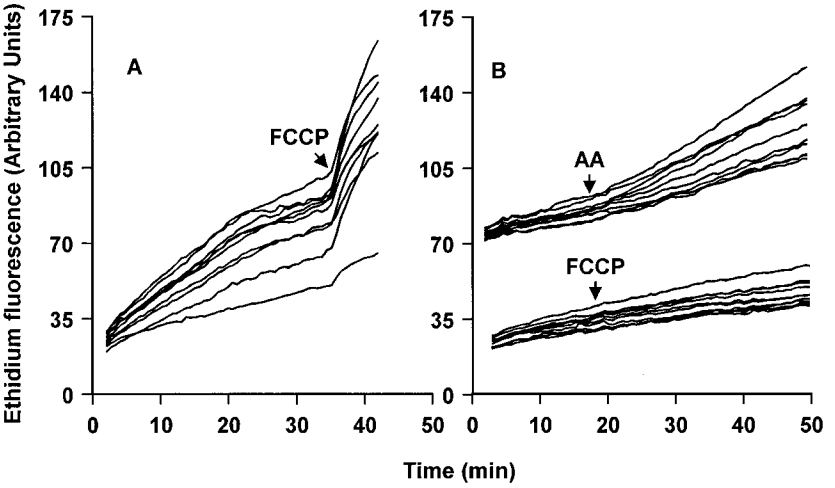

Fig. 4. Low concentrations of HET allow observation of $O_2^{\bullet-}$ production independent of a $\Delta\psi_m$-dependent artifact. A: Addition of protonophore (1 μM FCCP) to cells incubated in the continuous presence of 10 μM HET results in a large enhancement of fluorescence. B: Cells incubated in parallel with 1 μM HET are insensitive to protonophore addition but show an increased rate of $O_2^{\bullet-}$ production in response to 1 μM antimycin A (AA). In both A and B 1 μg/ml oligomycin is also present throughout to ensure a complete reduction in $\Delta\psi_m$.

tives react with H_2O_2 to give oxidised products which are pH dependent and to varying extents cell permeant [9,21]; despite difficulties in deconvoluting the pH effect using this indicator, protonophores have been reported to inhibit generation of reactive oxygen species in cortical neurones [13]. Dihydrorhodamine-123 responds also to H_2O_2 [21] and peroxynitrite [22,23] and the product rhodamine-123 is again a mitochondrial $\Delta\psi_m$ indicator; using this indicator protonophores were reported to double production of reactive oxygen species in cortical neurones [10]. In a comprehensive study to establish that HET reacted selectively with $O_2^{\bullet-}$ in cultured hippocampal neurones, Bindokas et al. [13] also reported a protonophore-induced enhancement in signal which was interpreted as increased $O_2^{\bullet-}$ generation.

The central goal of many related studies is to establish the chain of causality between physiological or pathological decreases in mitochondrial polarisation, $O_2^{\bullet-}$ generation, the mitochondrial permeability transition and subsequent apoptotic and necrotic cell death (reviewed in [1,7]). Two major areas where HET has been employed to report a correlation between a decreased $\Delta\psi_m$ and an enhanced $O_2^{\bullet-}$ generation are in dexamethasone-induced lymphocyte apoptosis [7] and neuronal glutamate excitotoxicity [13]. We feel that some of these conclusions should be re-examined to deconvolute the absolute production of ethidium from the variable fluorescent enhancement depending on compartmentation of this mitochondrial membrane potential indicator [16].

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References

[1] Skulachev, V.P. (1996) Q. Rev. Biophys. 29, 169–202.
[2] Turrens, J.F., Alexandre, A. and Lehninger, A.L. (1985) Arch. Biochem. Biophys. 237, 408–414.
[3] Van Belzen, R., Kotlyar, A.B., Moon, N., Dunham, W.R. and Albracht, S.P.J. (1997) Biochemistry 36, 886–893.
[4] Liu, S.S. and Huang, J.P. (1996) in: Procedings of the International Symposium on Natural Antioxidants (Moores, D., Ed.) AOCS Press, Champaign, IL (in press).
[5] Boveris, A. and Chance, B. (1973) Biochem. J. 128, 617–630.
[6] Golstein, P. (1997) Science 275, 1081–1082.
[7] Petit, P.X., Susin, S.A., Zamzami, N., Mignotte, B. and Kroemer, G. (1996) FEBS Lett. 396, 7–13.
[8] Richter, C., Schweizer, M., Cossarizza, A. and Franceschi, C. (1996) FEBS Lett. 378, 107–110.
[9] Reynolds, I.J. and Hastings, T.G. (1995) J. Neurosci. 15, 3318–3327.
[10] Dugan, L.L., Sensi, S.L., Canzoniero, L.M.T., Handran, S.D., Rothman, S.M., Lin, T.S., Goldberg, M.P. and Choi, D.W. (1995) J. Neurosci. 15, 6377–6388.
[11] Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B. and Kroemer, G. (1995) J. Exp. Med. 182, 367–377.
[12] Castedo, M., Macho, A., Zamzami, N., Hirsch, T., Marchetti, P., Uriel, J. and Kroemer, G. (1995) Eur. J. Immunol. 25, 3277–3284.
[13] Bindokas, V.P., Jordan, J., Lee, C.C. and Miller, R.J. (1996) J. Neurosci. 16, 1324–1336.
[14] Scanlon, J.M., Aizenman, E. and Reynolds, I.J. (1997) Eur. J. Pharmacol. 326, 67–74.
[15] Macho, A., Castedo, M., Marchetti, P., Aguilar, J.J., Decaudin, D., Zamzami, N., Girard, P.M., Uriel, J. and Kroemer, G. (1995) Blood 86, 2481–2487.
[16] Rottenberg, H. (1984) J. Membr. Biol. 81, 127–138.
[17] Courtney, M.J., Lambert, J.J. and Nicholls, D.G. (1990) J. Neurosci. 10, 3873–3879.
[18] Budd, S.L. and Nicholls, D.G. (1996) J. Neurochem. 66, 403–410.
[19] Budd, S.L. and Nicholls, D.G. (1996) J. Neurochem. 67, 2281–2291.
[20] Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 192, 873–880.
[21] Royall, J.A. and Ischiropoulos, H. (1993) Arch. Biochem. Biophys. 302, 348–355.
[22] Henderson, L.M. and Chappell, J.B. (1993) Eur. J. Biochem. 217, 973–980.
[23] Kooy, N.W., Royall, J.A., Ischiropoulos, H. and Beckman, J.S. (1994) Free Radical Biol. Med. 16, 149–156.