Mitochondrial function is required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*

Chris M. Grant*, Fiona H. MacIver, Ian W. Dawes

C.R.C. for Food Industry Innovation, School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, N.S.W. 2052, Australia

Received 2 May 1997

Abstract **Yeast strains that lack mitochondrial function are sensitive to oxidative stress caused by reactive oxygen species (ROS). Specifically, rho^0 mutants that lack mitochondrial DNA, and strains deleted for the nuclear genes *COX6* and *COQ3* that are required for function of the respiratory electron transport chain, were sensitive to H_2O_2. In addition, treatment with mitochondrial inhibitors including antimycin A, oligomycin, potassium cyanide and sodium azide increased sensitivity to H_2O_2. The mechanism does not appear to depend on the antioxidant status of the cell since respiratory-deficient strains were able to mount an inducible adaptive response to H_2O_2. We suggest that the oxidant sensitivity is due to a defect in an energy-requiring process that is needed for detoxification of ROS or for the repair of oxidatively damaged molecules.**

Key words: Oxidative stress; Respiratory deficient; Respiration; Coq3; Cox6; *Saccharomyces cerevisiae*

1. Introduction

Cells are exposed to a range of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{-\bullet}$) and the hydroxyl radical ($^\bullet OH$) during the course of normal aerobic metabolism and following exposure to radical-generating compounds. Such ROS can damage a wide range of macromolecules in the cell including nucleic acids, proteins and lipids, eventually leading to cell death. However, cells contain various antioxidant defense mechanisms to protect against the toxic effects of ROS, including enzymes such as catalase, glutathione peroxidase and superoxide dismutase, as well as non-enzymic molecules such as glutathione, uric acid, and vitamins C and E which can bind and inactivate ROS directly [1,2]. During oxidative stress, a proportion of the ROS evade or overcome the host defenses resulting in oxidative damage to cells [3,4].

In response to an oxidant challenge, yeast cells increase the synthesis of a number of genes involved in the detoxification of ROS including *GLR1* [5], *CTT1* [6], *TRX2* [7] and *GSH1* [8]. This increased synthesis of antioxidants forms the basis of the adaptive response in which treatment with low amounts of oxidant induces resistance to subsequent and otherwise lethal doses. Adaptive responses have been demonstrated for both H_2O_2 and the superoxide anion, and these stress responses appear to be distinct to that for the heat shock response [9–11]. More recently, yeast cells have also been found to adapt to the reactive aldehyde malondialdehyde generally formed as a consequence of lipid peroxidation [12]. Whilst there appears to be considerable overlap in the types of stress molecules induced by different oxidant treatments, cross adaption studies indicate that are also distinct responses to H_2O_2, the superoxide anion and malondialdehyde [10,12].

During respiration, mitochondria are the primary source of ROS in the cell. Molecular oxygen is relatively unreactive and harmless in its ground state, but can undergo partial reduction to form a number of ROS including the superoxide anion and H_2O_2 which can further react to produce the highly reactive hydroxyl radical [4]. Mitochondria are the major sources of the superoxide anion in the cell, and in vitro studies indicate that complex III of the respiratory chain may be responsible for more than 80% of this ROS produced in yeast [13,14]. The role of respiration in ROS production in yeast has been further demonstrated by the observation that cells with a disruption of *SOD2*, encoding the mitochondrial superoxide dismutase, can grow under conditions of hyperoxia as a result of mutations which disrupt the electron transport chain [15]. In addition, the viability of strains lacking both *SOD1*, encoding the cytoplasmic superoxide dismutase, and *SOD2*, during long term stationary phase incubation can be restored by mutations which disrupt the electron transport chain [16]. These results further implicate the electron transport chain as a major source of ROS in yeast, since the absence of respiratory function restores the viability of strains affected in superoxide dismutase activity, which are normally sensitive to oxidants.

Given that the respiratory chain of the mitochondrion is an endogenous source of ROS, it is surprising that respiratory deficient (petite) yeast cells have been found to be sensitive to conditions of oxidative stress. Petite strains were reported to be sensitive to H_2O_2 [9,11], the superoxide anion [10,11] a product of lipid peroxidation [12] and the anti-tumor drug bleomycin [17]. Here, we report that this oxidant sensitivity of respiratory deficient strains arises as a result of a defect of respiratory function, presumably affecting an energy requiring process, rather than any difference in antioxidant capacity.

2. Materials and methods

2.1. Yeast strains and media

The *Saccharomyces cerevisiae* strains used in this study were CY4 (*MATa ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*) and its isogenic derivative CY4p (petite) generated by treatment with ethidium bromide [18]. Null alleles of *COX6* and *COQ3* were generated by a one step PCR amplification protocol that replaced the entire open reading frames with the yeast *HIS3* gene [19].

Strains were grown in rich YEPD medium (2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract) or minimal SD medium (0.17% (w/v) yeast nitrogen base without amino acids, 5% (w/v) ammonium sulfate, 2% (w/v) glucose) supplemented with appropriate amino acids and bases. For growth on non-fermentable carbon sources, YEPGE contained 3% (v/v) glycerol and 1% (v/v) ethanol. Media

*Corresponding author. Fax: (61) (2) 9385-1050.
E-mail: c.grant@unsw.edu.au

PII S0014-5793(97)00592-9

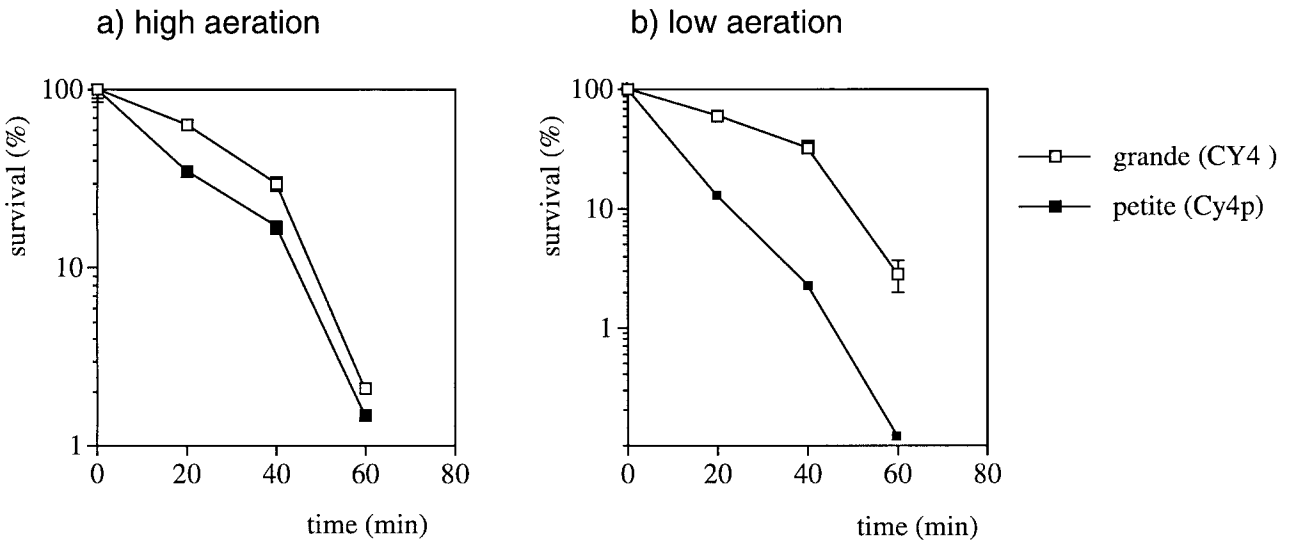

Fig. 1. Effect of respiratory competence on sensitivity to H_2O_2. Strains CY4 (grande) and CY4p (petite) were grown to exponential phase in YEPD ((1–2)×10^7 cells/ml) and treated with 4 mM H_2O_2 for 1 h under conditions of high aeration (a) and low aeration (b). Cells were diluted and plated on YEPD to monitor cell viability. Percentage survival is expressed relative to untreated control cultures (100%). Values shown are the mean of at least three determinations with error bars representing standard deviations.

were solidified by the addition of 2% (w/v) agar. For high aeration conditions cells were grown in 40 ml of media in 250 ml flasks with shaking at 220 rev/min, whereas, for growth under low aeration conditions, cells were grown in 5 ml of media in 10 ml centrifuge tubes on a tube roller at 80 rev/min [16].

2.2. Sensitivity to hydrogen peroxide

Dose–response curves were obtained by growing cells to exponential phase ((1–2)×10^7 cells ml^{-1}) in YEPD medium at 30°C, and treating with 4 mM H_2O_2 for 1 h. Aliquots of cells were diluted in fresh YEPD medium at 20-min intervals and plated in triplicate on YEPD plates to obtain viable counts after 3 days growth. For treatment with inhibitors of mitochondrial function including oligomycin, antimycin A, potassium cyanide and sodium azide, cells were grown in SD minimal media for 30 min prior to treatment with H_2O_2.

3. Results

3.1. Sensitivity of a respiratory incompetent yeast strain to hydrogen peroxide

To confirm that the strain background used in this study resulted in different sensitivities to H_2O_2 depending on respiratory ability, wild-type (CY4) and an isogenic rho^0 petite strain (CY4p), generated by treatment with ethidium bromide [18], were tested for sensitivity to hydrogen peroxide. Strains were grown to exponential phase (approximately 2×10^7 cells/ml) in YEPD medium and exposed to 4 mM H_2O_2 for 1 h during which cell survival was monitored by measuring viable counts. It is not yet clear whether cells respond to the H_2O_2 directly, or to some toxic reaction product generated in the

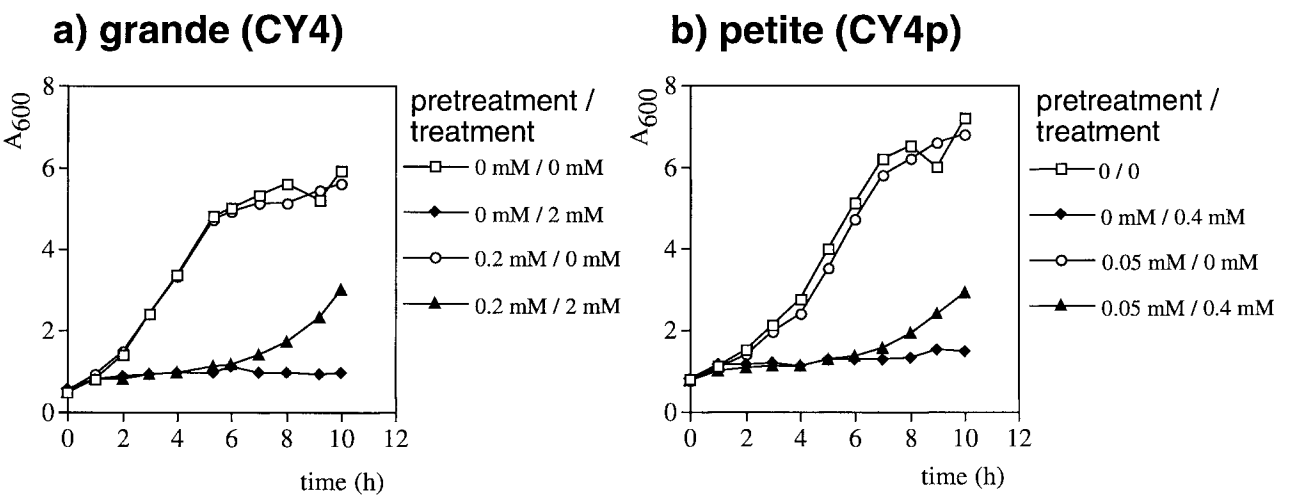

Fig. 2. Induction of H_2O_2 tolerance under low aeration growth conditions. (a) Strain CY4 (grande) was either pretreated with 0.2 mM H_2O_2 for 1 h (non-lethal pretreatment) then challenged with 2 mM H_2O_2 to test for lethality, or exposed to 2 mM H_2O_2 without pretreatment. (b) Strain CY4p (petite) was either pretreated with 0.05 mM H_2O_2 for 1 h (non-lethal pretreatment) then challenged with 0.4 mM H_2O_2 to test for lethality, or exposed to 0.4 mM H_2O_2 without pretreatment. Pretreatments were added at $t=0$, and treatments were added at $t=1$ h.

media [20]. However, previous studies have shown similar effects are observed when cells are treated with hydrogen peroxide in either complex media or phosphate buffer [9,11,12,21]. Under conditions of high aeration (normoxia) the petite strain was more sensitive to H_2O_2 than the grande strain (Fig. 1a). This difference in sensitivity was even more pronounced in cells grown under conditions of low aeration, which resulted in 2.8% survival for the grande strain compared to 0.12% survival for the petite strain (Fig. 1b). To determine whether the difference in oxidant sensitivity between the grande and petite strains was due to any differences in antioxidant capacity, the ability to mount an adaptive response to H_2O_2 was determined.

3.2. *Respiratory deficient strains mount an adaptive response to hydrogen peroxide.*

The ability of the petite mutant to mount an adaptive response to H_2O_2 was tested under conditions of low aeration in which the greatest difference in sensitivity between the grande and petite strains was observed. Sublethal doses of 0.2 mM and 0.05 mM H_2O_2 were administered to the grande and petite strains for 1 h, respectively. A lower concentration was used for the petite strain due to its high sensitivity to H_2O_2. Following this treatment, lethal concentrations of 2 mM and 0.4 mM H_2O_2 were administered to the grande and petite strains, respectively. These lethal concentrations prevented growth of both strains for up to 9 h (Fig. 2). However, the pretreatments resulted in restoration of growth after 5 h exposure to the lethal concentrations of H_2O_2. These results indicate that while petite strains differ from grande ones in their sensitivity, both strains can still adapt to become more resistant after exposure to H_2O_2. The difference between the between grande and petite strains is therefore unlikely to be due to the cells ability to adapt. However, the petite strain may have been more sensitive to H_2O_2 due to some lack of

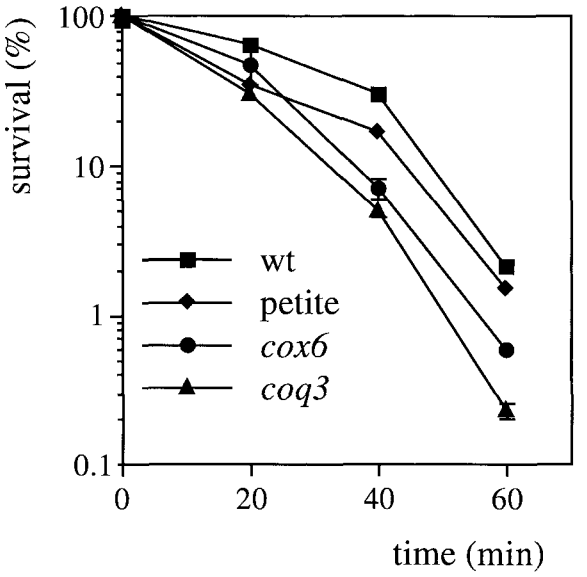

Fig. 3. Strains deleted for *COQ3* and *COX6* are sensitive to H_2O_2. The wild-type strain CY4 (grande) and *coq3*, *cox6* and petite (CY4p) mutants were grown to exponential phase in YEPD ($1–2\times10^7$ cells/ml) and treated with 4 mM H_2O_2 for 1 h. Cells were diluted and plated on YEPD to monitor cell viability. Percentage survival is expressed relative to untreated control cultures (100%).

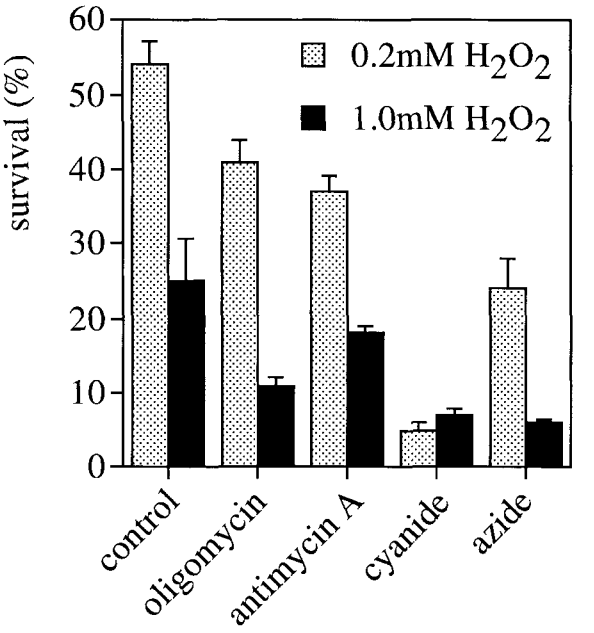

Fig. 4. Effect of mitochondrial inhibitors on resistance to H_2O_2. Cells were grown in minimal SD media to early exponential phase and treatment with water (control), 5 μg/ml oligomycin, 0.1 mM antimycin A, 0.5 mM potassium cyanide, or 0.5 mM sodium azide for 30 min. Cells were then challenged with 0.2 mM or 1.0 mM H_2O_2 for 1 h prior to plating on YEPD to monitor cell viability. Percentage survival is expressed relative to untreated control cultures (100%).

mitochondrially-related antioxidant capacity, or to a more general function of the mitochondria such as the potential for high-level energy production. The former possibility is difficult to test directly given the number of possible antioxidants, although we have shown that reduced glutathione levels are the same in both grande and petite strains [22]. Obviously many other cellular antioxidant systems remain to be tested. We therefore decided to examine the effects of disrupting specific mitochondrial functions, especially those involved in energy generation via the respiratory chain.

3.3. *Disruption of the respiratory electron transport chain results in sensitivity to hydrogen peroxide*

Strains deleted for *COQ3* and *COX6* were constructed to examine the peroxide sensitivity of respiratory deficient strains which arise due to defects in the electron transport chain. The *COQ3* gene encodes 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase and *coq3* mutants lack ubiquinone [23]. *COX6* encodes subunit VI of cytochrome-c-oxidase and *cox6* mutants are deficient in the terminal step in electron transport [15]. The *coq3* and *cox6* mutants were similar to the ethidium bromide generated rho^0 strain in that they showed greater sensitivity to H_2O_2 than the isogenic grande strain (Fig. 3).

We next examined the effect of disrupting the respiratory chain using the mitochondrial inhibitors oligomycin, antimycin A, potassium cyanide and sodium azide. The antibiotic oligomycin binds directly to the mitochondrial ATP synthase and inhibits both electron transfer and ATP production. Antimycin blocks electron flow at complex III and sodium azide and potassium cyanide block electron flow at complex IV of the respiratory electron transport chain preventing ATP pro-

duction [22]. Wild-type cells were grown to early exponential phase in minimal medium (A_{600}=0.5) prior to treatment with the various inhibitors for 30 min. Cells were then challenged with H_2O_2 for 1 h and cell survival determined. Control cells, pretreated with water alone, lost 46% and 75% viability following treatment with 0.2 mM and 1.0 mM, respectively (Fig. 4). For all of the mitochondrial inhibitors tested, there was a greater loss of viability following treatment with these concentrations of H_2O_2. For the case of potassium cyanide, there was only 5% and 7% survival following the treatments with 0.2 mM and 1.0 mM H_2O_2.

4. Discussion

Mitochondria are the primary source of ATP in nucleated cells via the process of oxidative phosphorylation. During this process, electrons are transported along four protein complexes that constitute the electron transport chain to the ultimate acceptor, molecular oxygen, with the formation of water. The resulting electrochemical proton motive force is used for the synthesis of ATP from ADP and phosphate. Leakage of electrons from the respiratory chain can result in the reduction of oxygen which is the primary source of ROS that damage various macromolecules in the cell. However, in this present study, we have shown that functional mitochondria are an essential component of the basal level resistance to oxidative stress caused by ROS. Thus, rho^0 mutants that lack mitochondrial DNA and hence cannot synthesize the mitochondrially-encoded components of the electron transport chain and mutants that lack the nuclear *COX6* and *COQ3* genes are sensitive to H_2O_2. Similarly, a wild-type strain treated with specific inhibitors of the respiratory chain showed elevated sensitivity to this oxidant.

Mitochondrial defenses against ROS in yeast include the enzymes Mn-superoxide dismutase, cytochrome-c-peroxidase and glutathione peroxidase [1,2,24]. These antioxidants are thought to be located in the mitochondria to guard against the free radical attack of mitochondrial lipids, nucleic acids and proteins (reviewed in [25]). There is also some evidence that respiratory growth can affect non-mitochondrial antioxidants since actively respiring cells are intrinsically more resistant to H_2O_2 and superoxide anions, and respiratory growth results in the transcriptional activation of various defense molecules [11]. Thus, the petite sensitivity to oxidants may be as a result of a defect in a mitochondrial or cellular antioxidant defense system. However, this appears unlikely since respiratory-defective strains are unaffected in their adaptive response to oxidants. This response is the result of a coordinated induction of antioxidant molecules leading to increased tolerance to ROS. Lack of mitochondrial function may however, lower basal level resistance to oxidants. In this view, the absence of respiration, and hence a lowered production ROS, would lead to a down-regulation of antioxidants. This model appears unlikely since the effect of mitochondrial inhibitors on wild-type cells was analogous to a complete loss of respiratory function. It is unlikely that treatment with these inhibitors would sufficiently lower ROS to down-regulate antioxidant expression.

The role of mitochondrial function in resistance to oxidants may be a requirement for ATP production in some energy-requiring process. This may for example, be an enzyme that repairs or removes oxidatively damaged molecules. Thus, the repair of damaged proteins or nucleic acids or the detoxification of lipid peroxidation products may be an energy requiring process. Alternatively, the active transport of oxidized and aberrant molecules from the cell or into the vacuole for subsequent breakdown reactions may also require energy. Future studies will aim to identify the energy-requiring process that accounts for the oxidant sensitivity of respiratory deficient strains.

References

[1] Grant, C.M. and Dawes, I.W. (1996) Redox Rep. 2, 223–229.
[2] Jamieson, D.J. (1995) Redox Rep. 1, 89–95.
[3] Sies, H. and de Groot, H. (1992) Toxicol. Lett. (Amsterdam) 64-65, 547–551.
[4] Halliwell, B. (1991) Am. J. Med. 91, 3C-14S–3C-38S.
[5] Grant, C.M., Collinson, L.P., Roe, J.-H. and Dawes, I.W. (1996) Mol. Microbiol. 21, 171–179.
[6] Marchler, G., Schüller, C., Adam, G. and Ruis, H. (1993) EMBO J. 12, 1997–2003.
[7] Kuge, S. and Jones, N. (1994) EMBO J. 13, 655–664.
[8] Stephen, D.W.S., Rivers, S.L. and Jamieson, D.J. (1995) Mol. Microbiol. 16, 415–423.
[9] Collinson, L.P. and Dawes, I.W. (1992) J. Gen. Microbiol. 138, 329–335.
[10] Flattery-O'Brien, J., Collinson, L.P. and Dawes, I.W. (1993) J. Gen. Microbiol. 139, 501–507.
[11] Jamieson, D.J. (1992) J. Bacteriol. 174, 6678–6681.
[12] Turton, H.E., Dawes, I.W. and Grant, C.M. (1997) J. Bacteriol. 179, 1096–1101.
[13] Boveris, A., Cadenas, E. (1982) in: Superoxide Dismutases, Vol. 2 (Oberley, L.W., Ed.), pp. 15–30, CRC Press, Boca Raton, FL.
[14] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.
[15] Guidot, D.M., McCord, J.M., Wright, R.M. and Repine, J.E. (1993) J. Biol. Chem. 268, 26699–26703.
[16] Longo, V.D., Gralla, E.D. and Valentine, J.S. (1996) J. Biol. Chem. 271, 12275–12280.
[17] He, C.H., Masson, J.-Y. and Ramotar, D. (1996) Curr. Genet. 30, 279–283.
[18] Grant, C.M., MacIver, F.H. and Dawes, I.W. (1996) Curr. Genet. 29, 511–515.
[19] Baudin, A., Ozier-Kalogeropoulos, O., Danouel, A., Lacroute, F. and Cullin, C. (1993) Nucleic Acids Res. 21, 3329–3330.
[20] Stephen, D.W.S. and Jamieson, D.J. (1997) Mol. Microbiol. 23, 203–210.
[21] Davies, J.M.S., Lowry, C.V. and Davies, K.J.A. (1995) Arch. Biochem. Biophys. 317, 1–6.
[22] Grant, C.M., MacIver, F.M. and Dawes, I.W. (1996) Mol. Microbiol. 22, 739–746.
[23] Clarke, C.F., Williams, W. and Teruya, J.H. (1991) J. Biol. Chem. 266, 16636–16644.
[24] Inoue, Y., Tran, L.-T., Kamakura, M., Izawa, S., Miki, T., Tsujimoto, Y. and Kimura, A. (1995) Biochim. Biophys. Acta 1245, 325–330.
[25] Richter, C., Schweizer, M. (1997) in: Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J.G., Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.