

Protective role of L-methionine against free radical damage of rat brain synaptosomes

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Incubation of rat brain synaptosomal/mitochondrial fraction with *tert*-butylhydroperoxide resulted in accumulation of the lipid peroxidation product, conjugated dienes, damage of the synaptosomal membrane as evidenced by leakage of lactate dehydrogenase, and decrease of the total content of glutathione and of the GSH/GSSG ratio. This treatment also produced a considerable decrease of the ouabain-sensitive ATPase activity and a much smaller diminution of the activities of glutathione reductase and glutathione transferase. Preincubation of the synaptosomal/mitochondrial fraction with 0.5 or 1.0 mM L-methionine significantly protected against lipid peroxidation, membrane damage and changes in the glutathione system produced by low (1 mM) concentrations of *tert*-butylhydroperoxide and completely prevented inactivation of ouabain-sensitive ATPase, glutathione reductase and glutathione transferase by such treatment. The importance of L-methionine in antioxidant protection is discussed.

Changes in the glutathione system and impairments of sodium and potassium transport are essential factors of brain tissue disturbance in various kinds of oxidative injury in the central nervous system such as Parkinson's and Alzheimer's diseases, ischaemia and reperfusion syndrome (Jenner, 1998; Draczynska-Lusiak *et al.*, 1998; Zeevalk *et al.*, 1998; Urano *et al.*, 1998; Canals *et al.*, 2001;

Keelan *et al.*, 2001). The glutathione system is responsible for neutralization of peroxides and maintenance of protein thiols in the reduced state (Cardoso *et al.*, 1998; Pocernich *et al.*, 2000). It is well known that neurons become more vulnerable to oxidative damages after a decrease in reduced glutathione content (Shukitt-Hale *et al.*, 1998). Studies on the glutathione system and sodium and potas-

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Abbreviation: TBH, *tert*-butylhydroperoxide.

sium ion transport under oxidative stress conditions make it possible (i) to understand the biochemical impairments occurring in the central nervous system and accompanying damages of the neurons, (ii) to study the ways of stabilization of the systems involved, which can be useful in the therapy of neurological diseases. The present approach is justified by the poor efficiency of traditional antioxidants in protecting the central nervous system against damage by free radicals.

We presumed that L-methionine may appear a positive modulator of the nervous system under oxidative stress conditions. This choice was made because this compound is an essential amino acid, necessary for the maintenance of nitrogen balance and growth of the organism and important for remethylation processes. L-Methionine neutralizes various toxic products by methylation and transsulphonation. Its lipotropic effect is related to its participation in choline synthesis. However, the interest in the antioxidative properties of this amino acid has started only recently (Bagchi *et al.*, 1998; De La Cruz *et al.*, 2000; Villalobos *et al.*, 2000).

The purpose of the present work was to study the effect of a free radical inducer, *tert*-butylhydroperoxide (TBH), on isolated nerve endings (synaptosomes) and to determine the possible protective properties of L-methionine.

MATERIALS AND METHODS

Biological material and treatment. The synaptosome-enriched fraction (called throughout as the synaptosomal/mitochondrial fraction) was obtained from large hemispheres of rat brain essentially according to the procedure of Weiler *et al.* (1981) as described previously (Chumakova & Liopo, 1996). In brief, albino rats (140–160 g) were killed by decapitation, the brains were quickly removed, the large hemispheres separated free of other fragments of the nervous system

and immediately placed in ice-cold isolation medium composed of 320 mM sucrose, 0.5 mM EDTA and 10 mM Tris/HCl (pH 7.4). After washing in this medium, the hemispheres were homogenized in the same cold medium, followed by centrifugation for 10 min at $1000 \times g$. The pellet was discarded and the supernatant was centrifuged for 20 min at $17\,000 \times g$. The resulting pellet was suspended in the same medium and was used throughout.

Exposure of the synaptosome-enriched fraction to oxidative stress was performed by suspending aliquots of the particles containing 0.6 mg protein in 0.5 ml of saline medium (150 mM NaCl, 5 mM KCl and 10 mM Tris/HCl, pH 7.4) and incubating for 30 min at 37°C with various concentrations of TBH. To examine the protective effect of L-methionine the particles were first preincubated with this amino acid for 30 min in the same medium and at the same temperature. Thereafter, the particles were sedimented by centrifugation for 3 min at $4000 \times g$, resuspended in the same medium and analysed as specified or incubated with TBH as indicated above.

Damage to the synaptosomal membrane was assessed by measuring the leakage of lactate dehydrogenase determined from the rate of NADH oxidation by pyruvate (Vasault, 1983). The total activity of lactate dehydrogenase was determined by lysing these particles in 0.2% Triton X-100.

Analytical procedures. Conjugated dienes were determined in heptane extracts at 235 nm (Recknagel & Ghoshal 1966). Reduced (GSH) and oxidized (GSSG) glutathione were measured enzymatically with methylglyoxal in the presence of glyoxalase and with NADPH in the presence of glutathione reductase, respectively (Akerboom & Sies, 1981). Protein thiol and disulphide groups were determined with the Ellman reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)], which forms colour complexes with -SH groups at pH 8.0 and with -SS- groups at pH 10.5, with maximum absorbance at 412 nm (Robyt *et al.*, 1971).

Determination of enzyme activities. Catalase activity was determined by the rate of H_2O_2 degradation recorded at 240 nm (Aebi, 1984). Glutathione reductase activity was measured by the rate of NADPH oxidation by oxidized glutathione (Carlberg & Mannervik, 1985). The assay for glutathione peroxidase was performed by measuring the formation of oxidized glutathione in a reaction between reduced glutathione and *tert*-butylhydroperoxide (Flohé & Günzler, 1984). Glutathione transferase activity was determined using reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates (Rice-Evans *et al.*, 1991). (Na^+, K^+) -ATPase was measured by determination of ouabain-sensitive liberation of inorganic phosphate (Foley, 1997).

Reagents. Glutathione reductase, glyoxalase, methylglyoxal, L-methionine, 5,5'-dithio-bis-(2-nitrobenzoic acid), *tert*-butylhydroperoxide, ATP and ouabain were from Sigma (St. Louis, MO, U.S.A.); NADH and NADPH were from Boehringer (Mannheim, Germany); NADP and lactate dehydrogenase were from Reanal (Budapest, Hungary).

Statistics. The results are presented as mean values \pm standard deviation (S.D.) for at least five experiments. Statistical significance was assessed using the Student's *t*-test, and the difference was admitted as significant at $P \leq 0.05$.

RESULTS

Effects of *tert*-butylhydroperoxide

Treatment of the synaptosomal/mitochondrial fraction with TBH produced typical features of an oxidative stress. The effects were concentration-dependent. Lipid peroxidation, determined as accumulation of conjugated dienes, is illustrated in Fig. 1 and the oxidative shift of the glutathione system is shown in Fig. 2. As can be calculated from the data of Fig. 2 (see also Table 1), the GSH/GSSG ratio decreased from the control value of 9.9 to 3.2

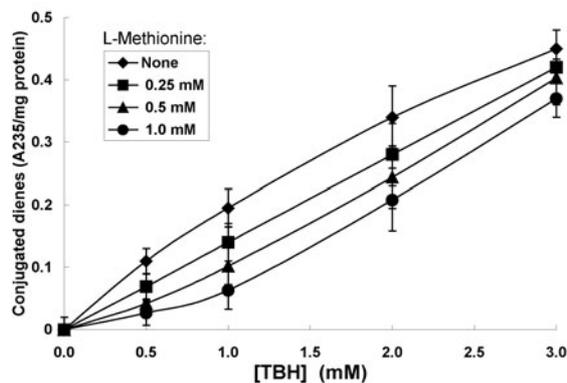


Figure 1. Effect of preincubation with L-methionine on the production of conjugated dienes in the synaptosomal/mitochondrial fraction under treatment with TBH.

The particles were preincubated with different concentrations of L-methionine, followed by incubation with various concentrations of TBH as described under Materials and Methods, and the production of conjugated dienes was determined.

after incubation with 1 mM TBH and to 0.9 after incubation with 3 mM TBH. It can also be seen that the total amount of glutathione decreased as well. Its content, calculated as $GSH + 2 \times GSSG$, decreased from 7.2 nmol/mg protein in the untreated preparations to 4.6 and 3.6 nmol/mg protein following 30 min incubation with 1 and 3 mM TBH, respectively.

Ouabain-sensitive ATPase appeared to be very sensitive to TBH. Its activity decreased below 50% after incubation with 1 mM TBH (Fig. 3). A similar decrease was observed in

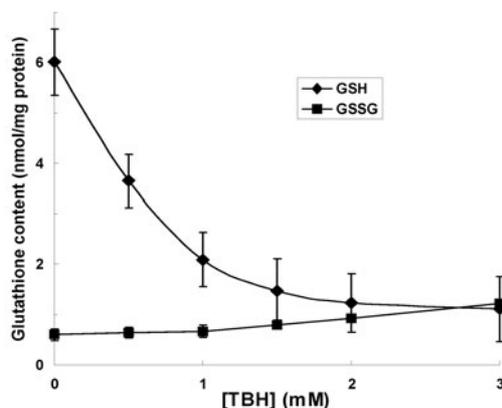


Figure 2. Effect of TBH on the content of reduced and oxidized glutathione in the synaptosomal/mitochondrial fraction.

Table 1. Effect of L-methionine and TBH on glutathione content and its redox state in the synaptosomal/mitochondrial fraction.

The particles were incubated with L-methionine and TBH as described under Materials and Methods. Control samples were incubated without these additions. Glutathione content is expressed in nmol/mg protein. Statistical significance: *, $P \leq 0.05$ compared to control samples; #, $P \leq 0.05$ compared to particles treated with TBH only.

	GSH	GSSG	GSH + 2 × GSSG	GSH/GSSG
Control	6.01 ± 0.32	0.61 ± 0.03	7.23 ± 0.35	9.92 ± 0.51
0.5 mM L-methionine	7.47 ± 0.41*	0.40 ± 0.04*	8.27 ± 0.41	18.19 ± 0.92*
1.0 mM L-methionine	7.43 ± 0.52*	0.31 ± 0.03*	8.05 ± 0.43	22.63 ± 0.95*
1.0 mM TBH	2.81 ± 0.21*	0.91 ± 0.04*	4.63 ± 0.47*	3.16 ± 0.25*
0.5 mM L-methionine + 1.0 mM TBH	3.72 ± 0.22*#	0.66 ± 0.05#	5.04 ± 0.45*	5.58 ± 0.32*#
1.0 mM L-methionine + 1.0 mM TBH	3.73 ± 0.22*#	0.40 ± 0.04*#	4.53 ± 0.38*	9.28 ± 0.42#

the content of protein thiol groups (Fig. 3), whereas the intactness of the synaptosomal membrane as evaluated from the leakiness of lactate dehydrogenase was increased by about 15% (Fig. 4). The activity of glutathione peroxidase was not changed after preincubation of the particle seven with 3 mM TBH, whereas the activities of glutathione transferase and glutathione reductase were significantly decreased (Fig. 5).

Effects of L-methionine

Preincubation of the synaptosomal/mitochondrial fraction with L-methionine considerably protected against lipid peroxidation by TBH. The strongest protective effect was observed against 0.5 and 1.0 mM TBH, whereas the protection against higher TBH concentrations was less pronounced (Fig. 1). L-Methionine also decreased the leakage of lactate dehydrogenase induced by 1 mM TBH from 16% to less than 9% (Fig. 4).

L-Methionine alone increased the reduction level of glutathione and slightly but not significantly increased the total glutathione content in the synaptosomal/mitochondrial fraction (Table 1). When the preincubation with L-methionine was followed by incubation with

TBH, a significant protective effect against the damage to the glutathione system was observed. The GSH/GSSG ratio was almost completely restored by 1 mM L-methionine, but the amount of GSH and the total content

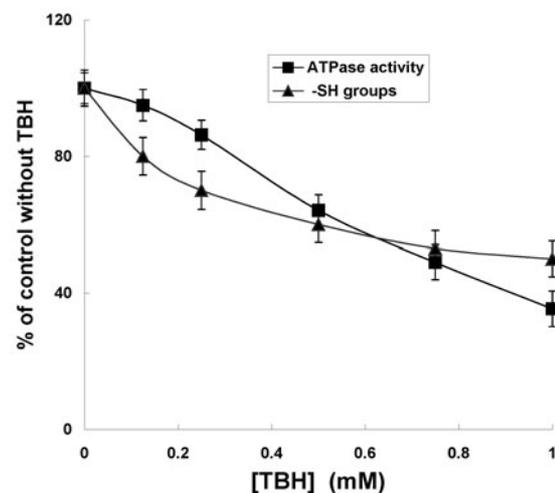


Figure 3. Effect of TBH on the content of -SH groups and the activity of ouabain-sensitive ATPase in the synaptosomal/mitochondrial fractions.

The content of -SH groups in the particles incubated without TBH (assumed as 100%) amounted to 50 ± 2 nmol/mg protein. The control (100%) activities of ATPase were: total, 5.78 ± 0.20 ; ouabain-insensitive, 4.27 ± 0.14 ; and ouabain-sensitive, 1.52 ± 0.10 μ mol/h per mg protein.

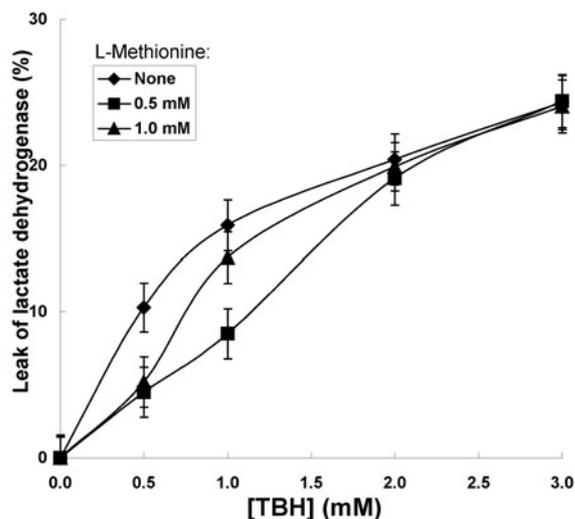


Figure 4. Protective effect of L-methionine against synaptosome damage by TBH treatment.

The synaptosomal/mitochondrial fraction was preincubated for 30 min without L-methionine or with two concentrations of this amino acid and then for another 30 min with different concentrations of TBH as described under Materials and Methods. Thereafter the particles were sedimented by centrifugation and lactate dehydrogenase activity was determined in the supernatants.

of glutathione were restored only partly (Table 1).

Preincubation with L-methionine appeared to have little effect on the activities of glutathione transferase and glutathione

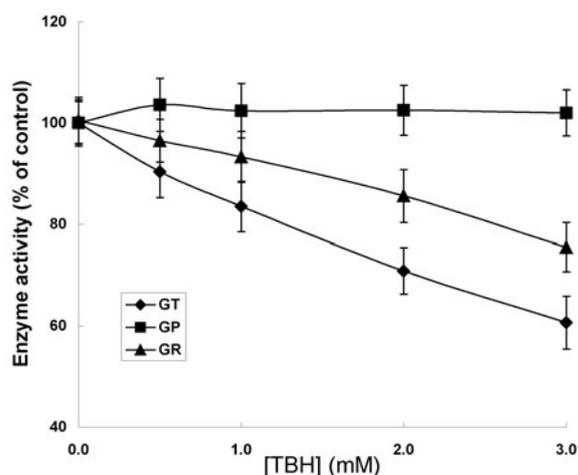


Figure 5. Effect of TBH on the activities of glutathione reductase (GR), glutathione peroxidase (GP) and glutathione transferase (GT) in the synaptosomal/mitochondrial fraction.

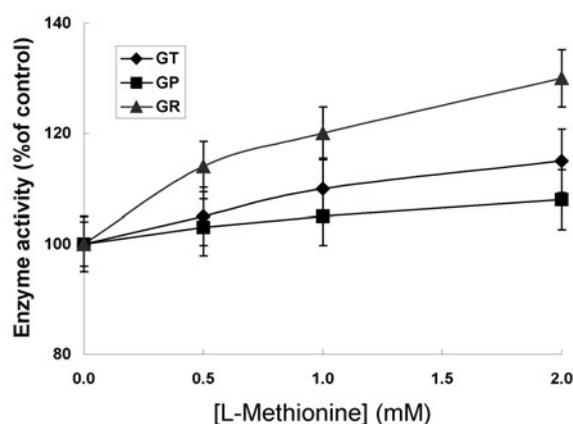


Figure 6. Effect of L-methionine on the activities of glutathione reductase (GR), glutathione peroxidase (GP) and glutathione transferase (GT) in the synaptosomal/mitochondrial fraction.

The experimental protocol was as described under Materials and Methods.

peroxidase and slightly increased the activity of glutathione reductase (Fig. 6). When the biological material was subsequently incubated with 1 mM TBH, preincubation with methionine significantly protected against the partial inactivation of glutathione transferase, augmented the increase of glutathione reductase but did not change the activity of glutathione peroxidase (Fig. 7; for the effect of TBH alone see Fig. 5).

The activity of ouabain-sensitive ATPase was significantly potentiated by L-methionine at concentrations of 0.5 and 1.0 mM (Fig. 8) but decreased after preincubation with concentrations of 2.0 mM and higher (not shown). L-Methionine at 0.5 and 1.0 mM completely protected this enzyme against inactivation by TBH (Fig. 8).

DISCUSSION

Damaging effects of reactive oxygen species on living systems are well documented. They include oxidative attack on vital cell constituents, such as proteins, lipids and nucleic acids (for reviews see Halliwell & Gutteridge, 1984;

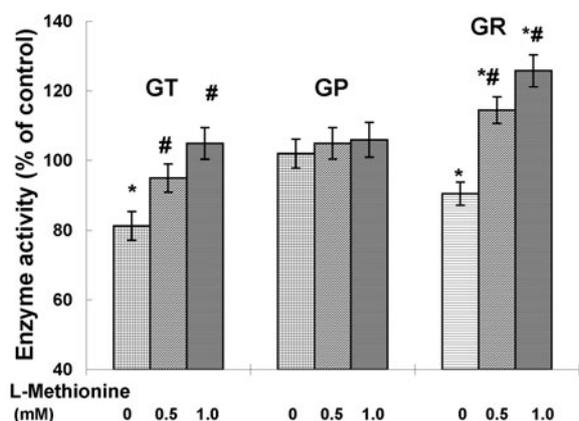


Figure 7. Combined effects of L-methionine and TBH on the activities of glutathione transferase (GT), glutathione peroxidase (GP) and glutathione reductase (GR).

The synaptosomal/mitochondrial fraction was first preincubated without or with L-methionine, followed by incubation with 1.0 mM TBH, as described in Materials and Methods, and the enzyme activities were determined. The values are expressed in percentage of those for the control samples incubated for the same period of time without L-methionine and TBH treatment. Statistical significance: *, $P \leq 0.05$ compared to control samples; #, $P \leq 0.05$ compared to particles treated with TBH only.

Farber, 1994; Martínez-Cayuela, 1995). In particular, membranous structures and their constituents, membrane-bound enzymes, are prone to oxidative damage. Plasma membrane (Na^+, K^+)-ATPase, whose activity is responsible for maintaining the balance between the major cell cations, Na^+ and K^+ , has long attracted attention as a possible target for oxygen free radicals. First reports on the inactivation of membrane-bound (Na^+, K^+)-ATPase in erythrocytes by superoxide radicals date from a quarter of a century ago (Bartosz *et al.*, 1977). Subsequent studies have pointed to a high susceptibility of (Na^+, K^+)-ATPase to oxidative inactivation in various tissues (see, e.g., Huang *et al.*, 1992; Reddy & Bhat, 1999) and, in particular, in brain neurons (Hexum & Fried, 1979; Arkhipenko *et al.*, 1985; Koide *et al.*, 1986; Palmer, 1987; Asano *et al.*, 1989; Hitschke *et al.*, 1994; Lehotsky *et al.*, 1999). This is in con-

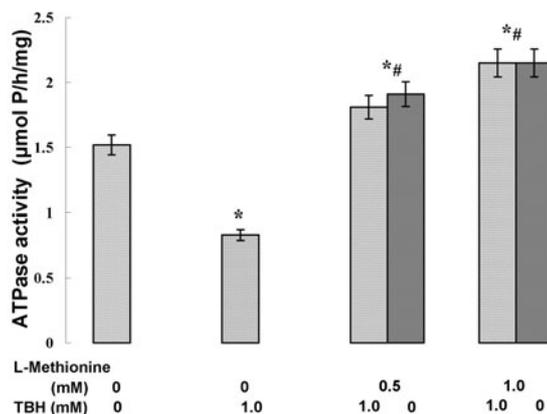


Figure 8. Effects of L-methionine and TBH on the activity of ouabain-sensitive ATPase in the synaptosomal/mitochondrial fraction.

The particles were preincubated for 30 min with various concentrations of L-methionine and thereafter incubated with 1.0 mM TBH. Statistical significance: *, $P \leq 0.05$ compared to control samples; #, $P \leq 0.05$ compared to particles treated with TBH only.

trast to the activation of (Na^+, K^+)-ATPase by oxidative stress in brain microvessels (Koide *et al.*, 1986; Asano *et al.*, 1989), the fact that may contribute to ischaemic evolution of brain edema.

The present results on the rat brain synaptosomal/mitochondrial fraction are fully in line with previous investigations. We confirmed the high susceptibility of synaptosomal (Na^+, K^+)-ATPase to peroxidative inactivation. In fact, after incubation of the particles with 1 mM TBH the activity of (Na^+, K^+)-ATPase was decreased by more than 60% (Fig. 3), whereas those of glutathione transferase and glutathione reductase by about 15% and 10%, respectively, and glutathione peroxidase remained unaffected (Fig. 5). The decrease of (Na^+, K^+)-ATPase activity was presumably due to the modification of -SH groups (Fig. 3) as already observed for the brain and kidney enzyme (Iwata *et al.*, 1988). We also observed damage of the brain synaptosomal membrane as manifested by increased leakage of lactate dehydrogenase (Fig. 4), which ran in parallel to the degree of lipid peroxidation, similarly as the previously

observed damage of the plasma membrane of Ehrlich ascites tumour cells (Slyshenkov *et al.*, 1995; 1996). As expected, incubation of the synaptosomal/mitochondrial fraction with TBH also resulted in drastic changes in the content and the redox state of glutathione. The total content of glutathione that could be determined by the enzymatic procedure employed ("free" glutathione) was decreased by half and the GSH/GSSG ratio to one third of its control value (Table 1 and Fig. 2). This points to an imbalance in the antioxidant defence system.

Apart from cysteine, L-methionine is another amino acid most vulnerable to oxidation (Vogt, 1995; Levine *et al.*, 1996; 1999; Berlett & Stadtman, 1997). Oxidation of methionine residues to methionine sulphoxide deprives respective proteins of their function as methyl donors and may be responsible for the loss of their biological activity (see, e.g., Carp *et al.*, 1982; Swaim & Pizzo, 1988; Ciorba *et al.*, 1997). On the other hand, methionine residues can act as powerful antioxidants (Levine *et al.*, 1996). A similar function can presumably be fulfilled by free L-methionine, as documented in the present work. L-Methionine not only partly protected against synaptosomal membrane damage (Fig. 4) and oxidative shift in the glutathione system (Table 1), but also completely prevented decrease of the (Na⁺,K⁺)-ATPase activity (Fig. 8). It also protected against slight decrease, produced by TBH, in the activities of glutathione transferase and glutathione reductase (Fig. 7). This protective effect was apparently due to an antioxidative mechanism, as manifested by a considerable reduction in the accumulation of lipid peroxidation products (Fig. 1). L-Methionine alone increased the reduction state of glutathione and also increased the total content of this tripeptide (Table 1), which can be interpreted as protection against endogenous peroxidative processes. It is most likely that free L-methionine, that accumulated within synaptosomes and mitochondria during the preincubation, played the

protective function, because the short preincubation time and the preincubation conditions, in particular the lack of energy source, were unfavourable for protein synthesis. Nevertheless, it cannot be excluded that some incorporation of methionine into proteins also occurred.

L-Methionine, both free and as a constituent of peptide chains, can be regarded as an excellent biological antioxidant, because its oxidation to methionine sulphoxide is reversible. The latter compound can be reduced back to methionine by methionine sulphoxide reductase, with thioredoxin serving as electron donor (Moskovitz *et al.*, 1999, 2001; Stadtman *et al.*, 2002). Thus, L-methionine, along with the GSH/GSSG system, forms an important link in the antioxidant defence system and contributes to the subtle prooxidant/antioxidant homeostasis within the cell.

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