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Downregulation of glutaredoxin but not glutathione loss leads to mitochondrial dysfunction in female mice CNS: Implications in excitotoxicity

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Abstract

Oxidative stress, excitotoxicity and mitochondrial dysfunction play synergistic roles in neurodegeneration. Maintenance of thiol homeostasis is important for normal mitochondrial function and dysregulation of protein thiol homeostasis by oxidative stress leads to mitochondrial dysfunction and neurodegeneration. We examined the critical roles played by the antioxidant, non-protein thiol, glutathione and related enzyme, glutaredoxin in maintaining mitochondrial function during excitotoxicity caused by β-*N*-oxalyl amino-L-alanine (L-BOAA), the causative factor of neurolathyrism, a motor neuron disease involving the pyramidal system. L-BOAA causes loss of GSH and inhibition of mitochondrial complex I in lumbosacral cord of male mice through oxidation of thiol groups, while female mice are resistant. Reducing GSH levels in female mice CNS by pretreatment with diethyl maleate or L-propargyl glycine did not result in inhibition of complex I activity, unlike male mice. Further, treatment of female mice depleted of GSH with L-BOAA did not induce inhibition of complex I indicating that GSH levels were not critical for maintaining complex I activity in female mice unlike their male counterpart. Glutaredoxin, a thiol disulfide oxidoreductase helps maintain redox status of proteins and downregulation of glutaredoxin results in loss of mitochondrial complex I activity. Female mice express higher levels of glutaredoxin in certain CNS regions and downregulation of glutaredoxin using antisense oligonucleotides sensitizes them to L-BOAA toxicity seen as mitochondrial complex I loss. Ovariectomy downregulates glutaredoxin and renders female mice vulnerable to L-BOAA toxicity as evidenced by activation of AP1, loss of GSH and complex I activity indicating the important role of glutaredoxin in neuroprotection. Estrogen protects against mitochondrial dysfunction caused by excitotoxicity by maintaining cellular redox status through higher constitutive expression of glutaredoxin in the CNS. Therapeutic interventions designed to upregulate glutaredoxin may offer neuroprotection against excitotoxicity in motor neurons.

Keywords: β-*N*-Oxalyl-amino-L-alanine; Complex I; Neurodegeneration; Estrogen; Oxidative stress; Motor neuron disease

1. Introduction

Brain has a high rate of metabolism and is dependent on energy supplies from mitochondria for normal functioning. Minor alterations in mitochondrial function can lead to oxidative damage and deleterious pathological changes in neurons (Dugan et al., 1995; Beal, 1995). Glutathione, a non-protein thiol plays an important role in the detoxification of reactive oxygen species in brain. The deficiency of glutathione leads to mitochondrial dysfunction (Sriram et al., 1998; Jha et al., 2000). Generally in brain (Shivkumar and Ravindranath, 1992; Shivkumar et al., 1995) and brain mitochondria in particular (Ravindranath and Reed, 1990) oxidative stress results in extensive formation of protein glutathione mixed disulfides (PrSSG) at available cysteine residues in proteins. Although glutathionylation of proteins is seen in certain cells of non-neural origin (Schuppe et al., 1992; Rokutan et al., 1994), it is more pronounced in brain, presumably because extrusion of GSSG, as routinely observed in other tissues such as lung and liver is not always seen in brain (Ravindranath and Reed, 1990; Shivkumar et al., 1995). Formation of PrSSG can potentially

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disrupt mitochondrial function by cross-linking sulfhydryl groups resulting in opening of the mitochondrial permeability transition pore, an event that can be blocked by the thiol reductant, dithiothreitol (Petronilli et al., 1994). These studies indicate that maintenance of thiol homeostasis is important for proper mitochondrial function.

Excitotoxic mechanisms have been proposed to play a role in the pathogenesis of various degenerative disorders (Tapia et al., 1999) including Huntington's, Parkinson's disease and amylotrophic lateral sclerosis (ALS). Mitochondria play a critical role in excitotoxic injury presumably by accumulating large amounts of calcium and generating reactive oxygen species (Lopachin, 1999). Thus, a close association and synergistic interplay exists between excitotoxicity, mitochondrial dysfunction, oxidative stress and neurodegeneration.

Glutaredoxin (also known as thioltransferase; Grx1), a cytosolic thiol disulfide oxidoreductase, specifically and efficiently reduces glutathionylated proteins to protein thiols utilizing reducing equivalents of NADPH (Gravina and Mieyal, 1993; Chrestensen et al., 2000). We have shown that Grx1 is essential for maintenance of mitochondrial complex I activity and downregulation of Grx1 results in loss of complex I activity (Kenchappa and Ravindranath, 2003) through oxidation of critical thiol groups in complex I subunits.

In the present study we have used an excitatory amino acid as a model neurotoxin to examine the relative contribution of GSH and Grx1 in maintaining mitochondrial function during toxic insult. β-*N*-Oxalyl amino-L-alanine (L-BOAA, also known as β-*N*-oxalyl-β-diamino propionic acid, β-ODAP; Spencer et al., 1986) is present in the chickling pea from the plant *Lathyrus sativus* and is known to be the causative factor for a type of motor neuron disease called neurolathyrism. Degeneration of anterior horn cells and loss of axons in the pyramidal tracts in lumbar spinal cord is seen in humans affected by neurolathyrism (Cohn and Streifler, 1981). L-BOAA is an agonist of the AMPA subtype of glutamate receptor (Pearson and Nunn, 1981; Ross et al., 1989). Oxidative stress and mitochondrial dysfunction, primary events in glutamate neurotoxicity (Schinder et al., 1996) are major contributors to L-BOAA induced toxicity. L-BOAA causes glutathione (GSH) loss and increase in protein glutathione mixed disulfides (PrSSG) in lumbosacral cord of male mice (Kenchappa et al., 2002) resulting in selective inhibition of mitochondrial complex I, a major component of the mitochondrial electron transport chain, due to oxidation of critical thiol groups (Sriram et al., 1998). In humans, the incidence of neurolathyrism is more common in men, while women are less prone to the disease although quantity of the chickling pea consumed is not significantly different (Roy, 1988; Norris et al., 1993). The animal model of L-BOAA toxicity mimics site-specificity and the gender difference seen in humans (Sriram et al., 1998). We have examined the molecular mechanisms underlying estrogen-mediated neuroprotection against mitochondrial dysfunction seen following L-BOAA administration with a view to understand the mechanisms underlying the pathogenesis of motor neuron loss in this disease with particular reference to protein thiol homeostasis since there are indications that glutaredoxin is regulated by estrogen in certain CNS regions (Kenchappa et al., 2004).

2. Experimental procedures

2.1. Materials

L-BOAA was obtained from Research Biochemicals (Natick, MA). Cysteinyl glutathione disulfide was purchased from Toronto Research Chemicals (Toronto, Canada). Ubiquinone 1 was obtained as a gift from Eisai Pharmaceutical Company (Tokyo, Japan). Northern blot analysis was performed using digoxigenin-labeling kit from Roche Biochemicals (Mannheim, Germany). Antibody to Grx1 was obtained from Lab Frontiers Life Science Institute (Seoul, Korea). All other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, MO) or Qualigens (Mumbai, India).

2.2. Animals

Male and female Swiss albino mice (1–3 months old) were obtained from the Animal Research Facility, National Institute of Mental Health and Neurosciences and National Brain Research Centre. Animals had free access to pelleted diet (Lipton, Calcutta, India) and water, *ad libitum*. All animal experiments were carried out according to National Institutes of Health guidelines for care and use of laboratory animals. All efforts were made to minimize animal suffering, to reduce number of animals used and to employ alternatives to *in vivo* techniques, if available. Female mice (3–4 weeks old) were anesthetized with ether and an incision of about 2–3 cm was made in the lower abdomen for ovariectomy. Ovaries were removed carefully and the abdomen was sutured. In sham-operated mice, a similar incision was made and the abdomen was sutured without removing ovaries. Ovariectomized and sham-operated mice were used for experimentation after 2 months. During sacrifice, atrophy of uterus and lack of ovaries were examined to ensure success of ovariectomy. Serum estrogen levels were measured using radioimmuno assay (Rao et al., 1984). L-BOAA (10 mg/kg body weight; s.c.) was dissolved in normal saline prior to use and animals were sacrificed 1 h later. In some experiments L-propargyl glycine (PPG; 100 mg/kg body weight) was administered to mice 6 h prior to L-BOAA and animals were sacrificed 1 h later. Diethylmaleate was administered subcutaneously at a dose of 430 mg/kg body weight and mice were sacrificed 2 h later. Control animals received vehicle alone.

2.3. Processing of tissue

Tissue was homogenized in 0.25 M sucrose and centrifuged at 1000 × *g* for 10 min to obtain post-nuclear supernatant, which was centrifuged again at 14,000 × *g* for 30 min to obtain the mitochondrial pellet. The post-mitochondrial supernatant was used for assay of Grx1 activity. Mitochondrial pellet was suspended in sucrose (0.25 M), and freeze-thawed for assay of complex I. The homogenate was centrifuged at 100,000 × *g* for 30 min to obtain cytosol and used for estimation of cystathionine-γ-lyase activity. For the assay of total GSH (GSH + GSSG) the frozen tissue was homogenized in 9 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. An aliquot of homogenate was added to an equal volume of 5-sulfosalicylic acid (10%, w/v), mixed and centrifuged at 10,000 × *g* for 10 min and the supernatant was used for estimation of total GSH. Protein concentration was estimated by a dye-binding method (Bradford, 1976).

2.4. Estimation of glutathione (GSH + GSSG)

Total glutathione (GSH + GSSG) was estimated by the enzymatic recycling method (Sriram et al., 1998). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) containing 0.8 mM dithiobisnitrobenzoic acid and 0.5 U/ml glutathione reductase in a total volume of 0.44 ml. The acid-soluble tissue supernatant (0.01 ml) prepared as described above was added. The reaction was initiated by adding 0.05 ml of 1.2 mM NADPH. The increase

in absorbance was measured at 412 nm over 3 min. A standard curve was generated using a known amount of GSSG.

2.5. Assay of cystathionine-γ-lyase

Cystathionine-γ-lyase activity was measured in cytosol prepared from mouse brain according to Heinonen (1973). The reaction mixture (total volume 0.5 ml) containing cytosolic protein from brain (2 mg), 100 μl Tris–HCl buffer (5 mM) and 10 μl of pyridoxal 5′-phosphate (1 mM) was considered as blank. The test sample contained all the above and 10 μl of L-cystathionine (2 mM), as substrate. Samples were incubated for 2 h and the reaction was terminated by placing the tubes in ice-water bath at 4 °C. Dithiothreitol (5 mM, 10 μl) was added to each tube to reduce all cystine. Ninhydrin reagent was added to all the tubes and heated in boiling water bath for 10 min. Tubes were cooled under tap water, the absorbance of the pink coloured complex was measured at 560 nm by spectrophotometric method (Gaitonde, 1967) using cysteine as standard.

2.6. Assay of NADH ubiquinone oxidoreductase (complex I) and Grx1 activities

Complex I activity was assayed in mitochondrial preparations as rotenone-sensitive NADH-ubiquinone oxidoreductase as described in detail (Sriram et al., 1998). Grx1 activity was estimated in post-mitochondrial supernatant using cysteinyl glutathione disulfide as substrate (Balijepalli et al., 1999).

2.7. Downregulation of Grx1 expression in mice using antisense oligonucleotides

Antisense and sense phosphorothionate end-capped oligonucleotides (21 mer) originating from the start codon of Grx1 cDNA (antisense: ATG GCT CAG GAG TTT GTG AAC; sense: TAC CGA GTC CTC AAA CAC TTA) were injected intrathecally into mice at 100 μg/dose, twice at 12 h intervals. The downregulation of Grx1 was examined by measuring enzyme activity 12 h after the last injection of oligonucleotides. L-BOAA (10 mg/kg body weight) was administered 11 h after the second injection of the end-capped oligonucleotides, and the animals were killed 1 h after L-BOAA. The lumbosacral segments of the spinal cord were dissected out and the activities of complex I and Grx1 were assayed as described earlier.

2.8. Northern blotting

Total RNA from lumbosacral cord of mice was extracted using TRI reagent. CNS regions from three animals were pooled for each sample preparation. Total RNA was separated electrophoretically and transferred to positively charged nylon membranes by capillary transfer, UV cross-linked and hybridized with digoxigenin-labeled cRNA prepared using the cDNA to Grx1 (Balijepalli et al., 1999). The blots were washed, incubated with antibody to digoxigenin conjugated with alkaline phosphatase and bands were visualized using chromogenic substrate for alkaline phosphatase. The relative intensity of the bands was estimated using densitometry.

2.9. Immunoblot analysis

Post-mitochondrial supernatant from lumbosacral cord was subjected to SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane, and incubated with the antibody to Grx1, followed by incubation with anti-rabbit IgG labeled with alkaline phosphatase. Immunostained bands were detected using nitroblue tetra zolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogens.

2.10. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from lumbosacral cord of vehicle and L-BOAA-treated, male, female, sham-operated and ovariectomized female mice (Korner et al., 1989). CNS regions from three animals were pooled for each sample preparation. Activation of AP1 was studied using EMSA as described in detail (Kenchappa et al., 2002).

Statistical analysis was performed using Student's *t*-test or ANOVA followed by Student–Newman Keul's or Dunnet's test as appropriate.

3. Results

3.1. Loss of GSH does not lead to mitochondrial dysfunction in female mice

Diethyl maleate (DEM) depletes GSH levels in cells through covalent modification of the thiol group in GSH thus rendering it unavailable as an anti-oxidant. Pretreatment of both male and female mice with DEM for 2 h decreased GSH levels significantly both in male and female mice cortex. The loss of GSH resulted in complex I inhibition only in male mice but not in female mice (Fig. 1a). Propargyl glycine (PPG) inhibits cystathionine γ-lyase (the rate-limiting enzyme in the conversion of methionine to cysteine) resulting in impaired synthesis of cysteine and decreased GSH levels. Pretreatment of mice with PPG results in lowered GSH levels in male and female mice cortex. However, the loss of complex I activity seen as a result of lowered GSH levels was again seen only in the male but not in the female mice (Fig. 1b). These results clearly indicate that GSH loss does not induce mitochondrial complex I dysfunction in female mice.

3.2. Differential response of male, female and ovariectomized mice to L-BOAA

We wanted to investigate if loss of GSH caused by prior inhibition of cystathionine γ-lyase by PPG conferred vulnerability to L-BOAA toxicity in female mice. Female mice were pretreated with PPG for 6 h followed by 1 h exposure of L-BOAA and GSH level was estimated in lumbosacral cord. PPG alone caused GSH loss (28%), but there was no loss of GSH following administration of L-BOAA alone indicating that female mice were resistant to L-BOAA induced GSH loss. Further, pretreatment with PPG followed by L-BOAA did not cause any additional loss of GSH in female mice lumbosacral cord (Fig. 2a). The administration of PPG and L-BOAA, alone or in combination did not affect the complex I activity indicating the resistance of female mice to L-BOAA toxicity (Fig. 2a).

In male mice a sustained decrease in complex I activity was seen from 15 min to 24 h in the lumbosacral cord following a single dose of L-BOAA (Fig. 2b) while it was unaffected in females. Ovariectomized mice were similar to male mice and complex I activity showed sustained inhibition in the lumbosacral cord (Fig. 2b). We also examined GSH levels after a single dose of L-BOAA. In ovariectomized mice, GSH levels decreased significantly 0.5 and 1 h after L-BOAA treatment (Fig. 2c) in a manner similar to that seen in male mice, while sham-operated female mice were unaffected. Basal GSH levels were similar in sham-operated and ovariectomized mice indicating that GSH levels were not influenced by estrogen. AP1 is a redox sensitive transcription factor whose

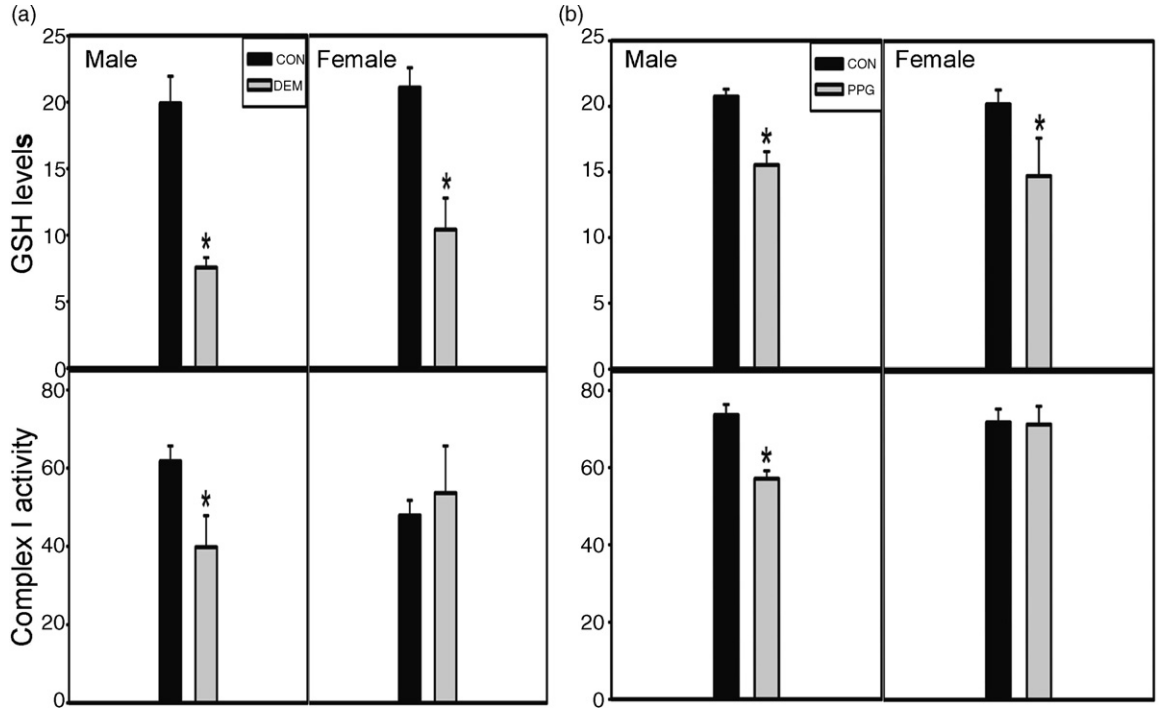

Fig. 1. Loss of GSH does not lead to mitochondrial dysfunction in female mice. Mice were pretreated for 2 h with diethyl maleate (430 mg/kg body weight, s.c.) or 6 h with L-propargyl glycine (100 mg/kg body weight, s.c.) and sacrificed later. Control animals received vehicle alone. GSH levels expressed as nanomoles GSH/mg protein and complex I activity expressed as nanomoles of NADH oxidized/min/mg protein were estimated in the cortex of male and female mice. Values are mean ± S.E.M. ($n = 3$ animals). Asterisk indicates values significantly different from controls ($p < 0.002$).

activity is modulated by agents that promote intracellular thiol perturbation (Galter et al., 1994). AP1 activation was significantly increased in lumbosacral cord of male and ovariectomized mice, 15 min after L-BOAA administration, whereas, it was unaffected in female mice (Fig. 2d). In ovariectomized mice, a series of events starting with AP1 activation, loss of GSH and complex I activity was seen, while they remained unchanged in sham-operated female.

3.3. Downregulation of Grx1 by antisense oligonucleotides in female mice results in loss of complex I

We downregulated Grx1 in female mouse brain by intrathecal administration of antisense phosphorothionate end-capped oligonucleotides (21 mer) originating from the start codon of Grx1 cDNA. This resulted in loss of Grx1 activity in lumbosacral cord (28% decrease) of female mice (Fig. 3). Downregulation of Grx1, per se resulted in loss of complex I indicating the Grx1 plays an important role to maintain complex I activity in female mice.

3.4. Female mice are sensitized to L-BOAA toxicity by downregulation of Grx1

Downregulation of Grx1 in female mice results in loss of complex I and sensitizes female mice to L-BOAA toxicity. In female mouse brain, intrathecal administration of antisense nucleotides resulted in 28% decrease in Grx1 activity in lumbosacral cord and abolished the neuroprotection seen in female mice against L-BOAA toxicity, as observed by the pronounced decrease in complex I activity in lumbosacral cord (Fig. 4).

3.5. Constitutive expression of Grx1 in male, female and ovariectomized mice

The basal level of Grx1 (measured as mRNA, protein and enzyme activity) was higher in the lumbosacral cord of female mice compared to males (Fig. 5a). Ovariectomy decreased the basal expression of Grx1 in lumbosacral cord indicating that the expression of Grx1 was regulated by estrogen (Fig. 5b–d). We examined the effect of ovariectomy on Grx1 activity in several CNS regions and non-CNS tissues, such as liver and kidney. Removal of ovaries resulted in significant loss of Grx1 activity in all the CNS regions examined, with the exception of thalamus and hippocampus (Fig. 5e). Further, Grx1 activity was unaffected in liver and kidney.

4. Discussion

Maintenance of protein thiol homeostasis is critical for mitochondrial complex I activity since it contains several cysteine residues (Dupuis et al., 1991) and facile glutathionylation of thiol groups in the 51 and 75 kDa subunits of complex by oxidants, such as GSSG results in inhibition of the enzyme activity (Taylor et al., 2003). The 75 kDa subunit of complex I is also a substrate for caspase 3 (Ricci et al., 2004). Glutathionylation of complex I subunits could result in

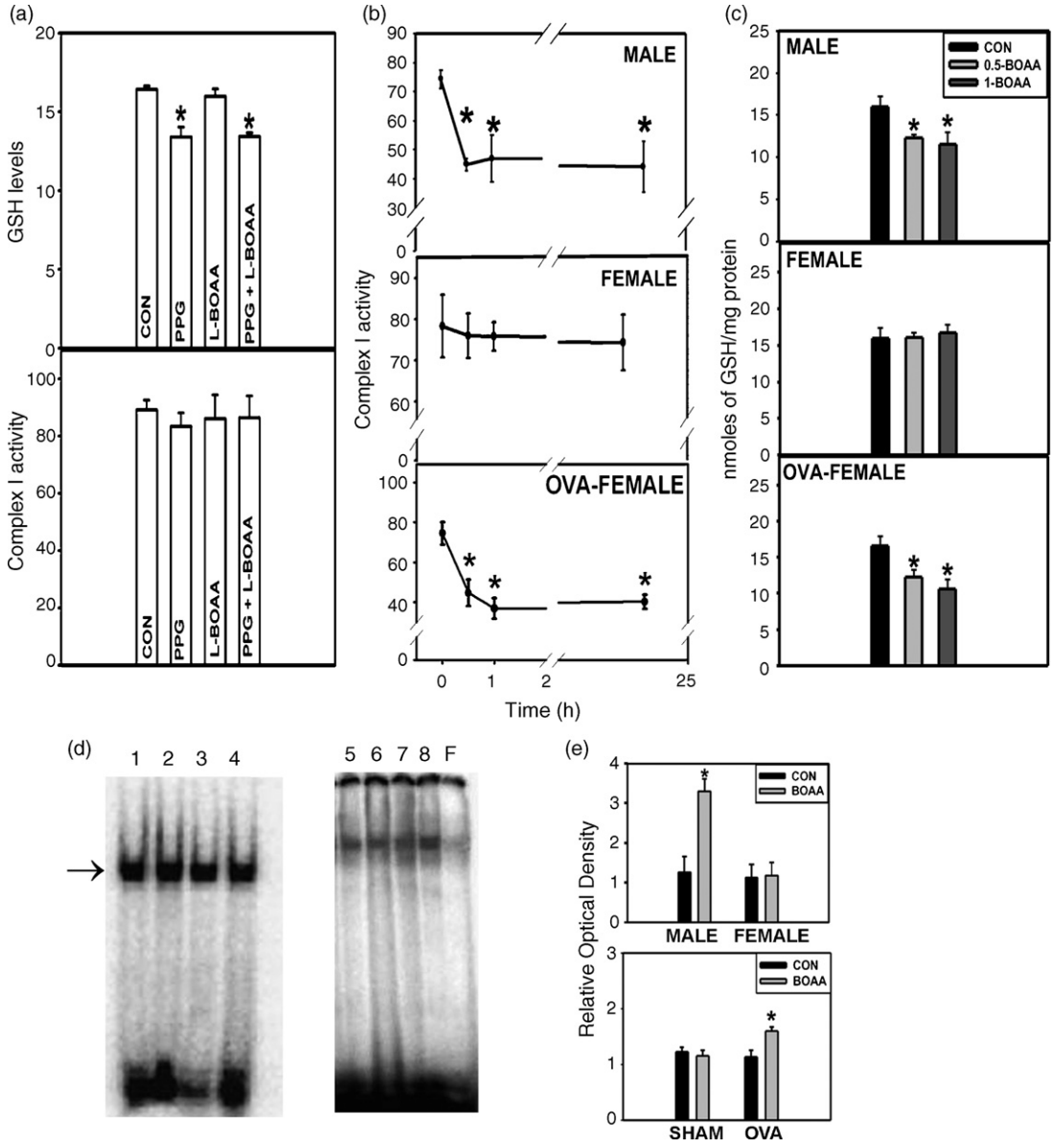

Fig. 2. Differential response of male, female and ovariectomized mice to L-BOAA. Female mice were pretreated for 6 h with L-propargyl glycine (100 mg/kg body weight, s.c.) prior to administration of L-BOAA (10 mg/kg body weight, s.c.) and sacrificed 1 h later. Control animals received vehicle alone. GSH levels as nanomoles of GSH/mg protein and complex I activity as nanomoles of NADH oxidized/min/mg protein were estimated in the lumbosacral cord of female mice. (a) Values are mean ± S.E.M. ($n = 3$ animals). Asterisk indicates values significantly different from controls ($p < 0.002$). Mice were administered single dose of L-BOAA and sacrificed after 0.5, 1 and 24 h. Complex I activity was estimated in lumbosacral cord of male, female and ovariectomized mice. Values are mean ± S.E.M. ($n = 6$ animals). Asterisk indicates values significantly different from corresponding vehicle treated controls ($p < 0.002$). (b) L-BOAA was administered and animals were killed 0.5 or 1 h later. (c) Total GSH (GSH + GSSG) levels were estimated in lumbosacral cord of male, female and ovariectomized mice. GSH levels are expressed as nanomoles of GSH/mg protein. Values are mean ± S.E.M. ($n = 6$ animals). Male, female, sham-operated and ovariectomized mice were administered a single dose of L-BOAA and sacrificed after 15 min. Electrophoretic mobility shift assay performed using nuclear extracts from lumbosacral cord. (d) Lanes 1 and 2 represent control and L-BOAA treated male mice, similarly lanes 3 and 4 represent control and L-BOAA treated female mice. Sham-operated control and L-BOAA treated female mice are represented in lanes 5 and 6 similarly ovariectomized mice are represented in lane 7 and lane 8. Lane containing only oligonucleotides without nuclear extract is represented in (F). (e) A representative densitometric analyses of EMSA are depicted from male and female, sham-operated and ovariectomized female mice treated with L-BOAA. The lanes in EMSA are represented in the same chronological order as shown in the densitometric quantitation. Values are mean ± S.E.M. ($n = 3$ individual experiments).

generation of increased reactive oxygen species, resulting in a vicious cycle of oxidative damage in mitochondria. Further, studies using PC12 cells have shown increased ROS leads to mitochondrial dysfunction and necrosis, indicating such mechanism may play a role in neurodegenerative disorders (Luo et al., 2005). Depletion of GSH in PC12 cells by downregulating γ-glutamyl cysteine synthetase (the rate-limiting enzyme for GSH synthesis) resulted in the loss of mitochondrial complex I activity but other components of the electron transport chain were unaffected (Davey et al., 1998; Jha et al., 2000). These studies have clearly demonstrated the selective vulnerability of mitochondrial complex I to oxidative

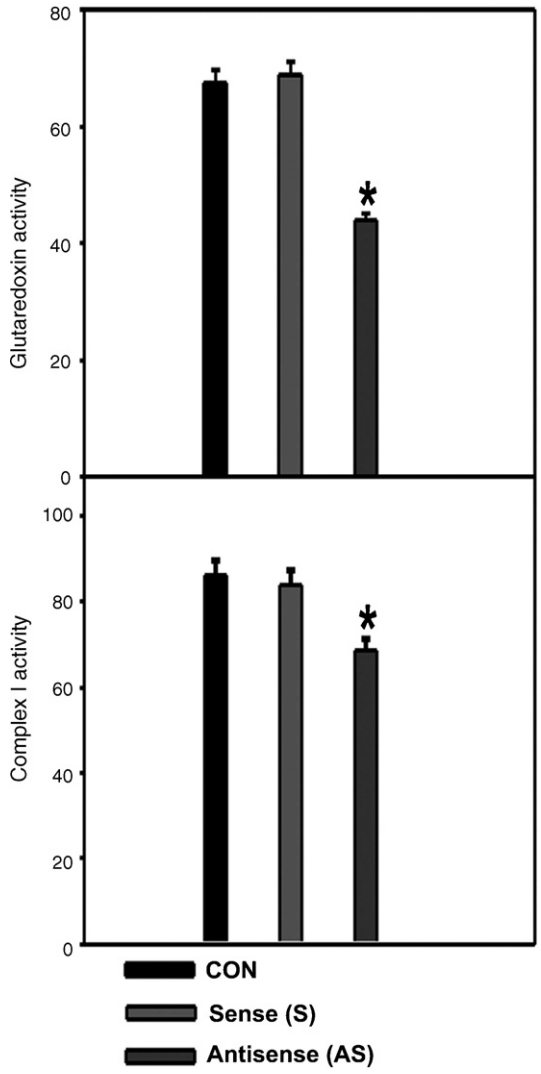

Fig. 3. Downregulation of Grx1 by antisense oligonucleotides in female mice results in loss of complex I activity. Female mice were treated with sense (S) or antisense (AS) oligonucleotides to Grx1 (200 μg) through intrathecal administration in two doses at 12 h intervals. Grx1 activity expressed as nanomoles of NADPH/min/mg protein and complex I activity expressed as nanomoles of NADH/min/mg protein were measured in lumbosacral cord. Values are mean ± S.D. ($n = 5$–6 animals). Asterisk (*) indicates values significantly different from vehicle-treated control ($p < 0.05$).

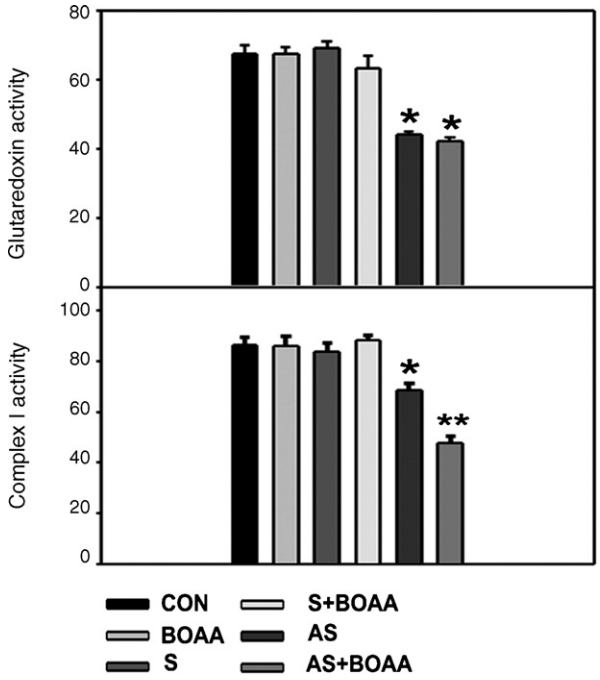

Fig. 4. Downregulation of Grx1 by antisense oligonucleotides sensitizes female mice to L-BOAA-mediated mitochondrial dysfunction. Female mice were treated with sense (S) or antisense (AS) oligonucleotides to Grx1 (200 μg) through intrathecal administration in two doses at 12 h intervals. L-BOAA was administered 11 h after the second dose and animals were killed 1 h later. Grx1 activity expressed as nanomoles of NADPH/min/mg protein and complex I activity expressed as nanomoles of NADH/min/mg protein were measured in lumbosacral cord. Values are mean ± S.D. ($n = 5$–6 animals). Asterisk (*) indicates values significantly different from vehicle-treated control, while asterisks (**) indicate values significantly different from the corresponding group of animals not treated with L-BOAA ($p < 0.05$).

modification through perturbation of thiol homeostasis. In the present study we examined the critical roles played by GSH vis-à-vis cytosolic Grx1 in maintaining mitochondrial function using the animal model of L-BOAA that not only causes selective toxicity in the motor cortex and lumbar sacral cord but also mimics the gender difference seen in humans.

Interestingly, in the present study we noticed that depletion of total GSH in female CNS did not result in mitochondrial dysfunction as seen in males. Loss of even 30% of GSH in lumbar sacral cord of males resulted in equivalent loss of complex I activity while depletion of up to 50% of GSH in females did not affect the enzyme activity. In contrast, in female mice downregulation of Grx1 (20% decrease) resulted in significant loss of complex I (30% loss), which was further potentiated when L-BOAA was co-administered (Figs. 3 and 4). Thus, Grx1 activity but not GSH levels seem critical for the neuroprotection seen in female mice. It is also possible that mitochondrial GSH levels are better maintained in female mice and are resistant to depletion compared to males. This may also contribute to the lack of inhibition of complex I in females following depletion of GSH, although constitutive gender difference is not seen in GSH levels.

Cysteine, the rate-limiting factor in GSH synthesis is made available to the neurons through the glutamate/cystine X_c^- antiporter and the glutamate EAAC1 transporter (Sato et al., 2005). Excess extracellular glutamate hinders the uptake of cystine leading to decreased GSH levels in neurons (Murphy et al., 1989) and EAAC1 knockout mice have lowered GSH levels (Aoyama et al., 2006). Thus, availability of cysteine is critical for maintaining GSH levels, which is also substantiated by the fact that inhibition of cystathionine γ-lyase by propargyl glycine leads to loss of GSH (Fig. 1; Diwakar and Ravindranath, 2007). The current study underscores the importance of Grx1, which effectively recycles GSH by reducing the mixed disulfides to the protein thiols. While loss of GSH in female mice has no effect on the neuroprotection, a small perturbation of Grx1 leads to mitochondrial dysfunction and renders them vulnerable to the excitotoxin. These studies

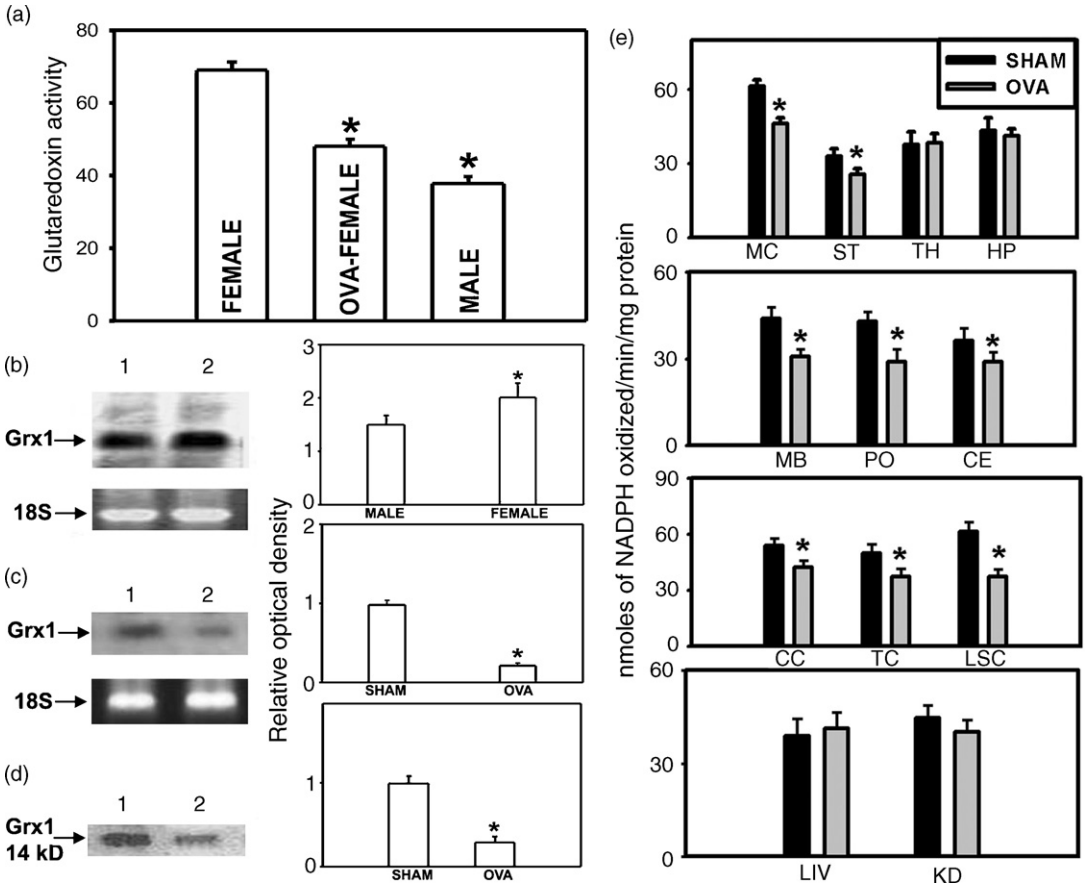

Fig. 5. Constitutive expression of Grx1 in male, female and ovarectomized mice. (a) Grx1 activity was estimated in lumbosacral cord of male, female and ovariectomized mice and expressed as nanomoles of NADPH/min/mg protein. Values are mean ± S.E.M. ($n = 6$ animals). (b) A representative Northern blot, corresponding 18S rRNA band of the total RNA and the densitometric analyses of the Northern blot representing relative intensity of hybridized bands from lumbosacral cord male and female mice are depicted. Each lane was loaded with 10 μg of total RNA. Values are mean ± S.E.M. ($n = 3$ individual experiments). (c) Represents a similar experiment as described above using ovariectomized and sham-operated female mice. Each lane was loaded with 15 μg of total RNA. Values are mean ± S.E.M. ($n = 3$ individual experiments). (d) A representative immunoblot blot and densitometric analyses of the immunoblot representing the relative intensity of the immunostained Grx1 protein in lumbosacral cord of ovariectomized and sham-operated female mice. Protein (15 μg) was loaded in each lane. The lanes are represented in the same chronological order as shown in the densitometric quantitation. Values are mean ± S.E.M. ($n = 3$ animals). Asterisk indicates values significantly different from corresponding controls ($p < 0.005$). (e) Grx1 activity was measured in motor cortex (MC), striatum (ST), cerebellum (CE), midbrain (MB), pons (PO), thalamus (TH), hippocampus (HP), cervical cord (CC), thoracic cord (TC), lumbosacral cord (LSC), liver (LIV) and kidney (KD) from sham-operated female (sham) and ovariectomized (ova) mice. Values are mean ± S.E.M. ($n = 6$ animals). Asterisk indicates values significantly different from sham-operated females ($p < 0.005$).

clearly demonstrate the importance of Grx1 in maintaining the functional integrity of the CNS.

Constitutively, Grx1 is expressed in higher levels in lumbosacral cord of female mice, the regions affected by L-BOAA (Fig. 5). Ovariectomy downregulates Grx1 in CNS regions affected by L-BOAA as well as in other regions, such as, striatum and cerebellum. However, in thalamus and hippocampus no difference is noted between sham-operated and ovariectomized animals indicating that Grx1 is not regulated by estrogen in these brain regions. Further, ovarietomy has no effect on Grx1 activity in liver and kidney indicating that it is not regulated by estrogen in these tissues. Individual brain regions respond very differently and potentially differential levels of Grx1 expression or turnover may contribute to determine the final outcome of estrogen-mediated neuroprotection, in addition to other factors. The antioxidant effect of estrogen in pharmacological doses has been demonstrated earlier, we now provide evidence, *in vivo*, of the regulation of expression of critical anti-oxidant enzymes, such as Grx1 by estrogen in CNS indicating that the neuroprotective effects of estrogen may be mediated through maintenance of redox status of critical proteins (Fig. 6).

Gender difference in Grx1 expression in lumbosacral cord was not completely abolished by ovariectomy. Although Grx1 activity decreased significantly in lumbosacral cord of female mice following ovariectomy (25% decrease compared to sham-operated females), it was higher than the activity seen in male mice. This may be due to the fact that serum estrogen levels in ovariectomized animals (10.0 ± 4.9 pg/ml serum) were reduced by 60% compared to the sham-operated female mice (27.0 ± 1.2 pg/ml serum). The presence of circulating estrogen synthesized by non-ovarian tissues such as, adrenal gland may account for the above.

Grx1 is a cytosolic enzyme and its role in maintaining mitochondrial function is as yet unclear. However, several

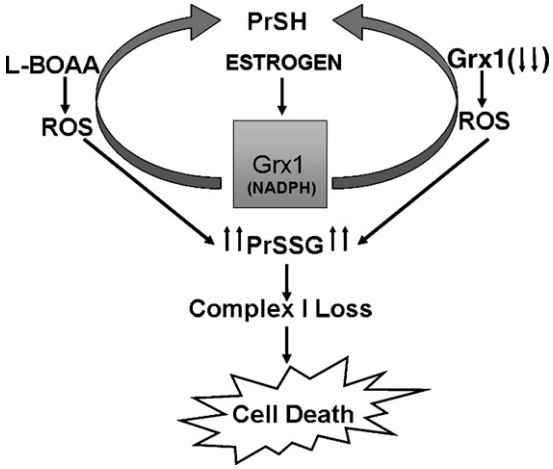

Fig. 6. Estrogen regulates Grx1 expression, which in turn maintains redox status of critical thiols of mitochondrial complex I. L-BOAA generates oxidative stress leading to protein thiol oxidation resulting in mitochondrial dysfunction seen as loss of complex I activity. Grx1 helps maintain the redox status of proteins and downregulation of Grx1 results in loss of complex I activity. Estrogen regulates Grx1 expression and upregulation of Grx1 by estrogen prevents mitochondrial dysfunction by maintaining redox status of critical thiol groups.

mitochondrial membrane proteins that are critically involved in maintenance of mitochondrial membrane potential (MMP) are sensitive to redox perturbation. For example, adenine nucleotide translocator (ANT), a specific carrier for exchange of adenosine triphosphate (ATP) and ADP is a major player in the maintenance of MMP. Oxidation of critical cysteine residue (cys 56) of ANT by thiol cross linkers leads to mitochondrial membrane permeabilization and cell death (Costantini et al., 2000). ANT also interacts with another component of outer membrane the voltage dependent anion channel (VDAC/porin). Alteration in mitochondrial membrane potential and opening of the mitochondrial permeability transition pore (White and Reynolds, 1996) through voltage-dependent channels is directly associated with an oxidation–reduction sensitive dithiol (Lieven et al., 2003), and the cross-linking of the sulfhydryl groups increases the probability of the mitochondrial permeability transition pore opening, an event that can be blocked by the thiol reductant, dithiothreitol (Petronilli et al., 1994; Vieira et al., 2000). Decrease in de-glutathionylation capability in the cytosol can potentially lead to perturbation of the redox status of other hitherto unidentified mitochondrial outer membrane proteins which may influence complex I activity.

The mitochondrial form of glutaredoxin (Grx2; Gladyshev et al., 2001; Lundberg et al., 2001) that shares only 36% sequence identity to Grx1 (Park and Levine, 1997) could also play a role in the reduction of glutathionylated complex I. While Grx1 seems to play an important role in preserving mitochondrial integrity thereby preventing complex I loss, Grx2 could potentially restore complex I activity in mitochondria through deglutathionylation. However, we did not find any gender difference in expression of Grx2 (data not shown).

Estrogen is known to regulate the expression of several neuroprotective genes. These include neurotrophic factors such as nerve growth factor, brain derived neurotrophic factor (BDNF), neurotrophin 3, their receptors TrkA–C and p75, and insulin-like growth factor 1 (IGF1) (Sohrabji et al., 1995; Toran-Allerand et al., 1999; Blurton-Jones et al., 1999). The promoter region of anti apoptotic genes, Bcl2 and $BclX_L$ contains ERE and estrogen enhances their transcription *in vitro* (Pike, 1999; Singer et al., 1999). A third group of genes regulated by estrogen are some structural proteins in neurons. Estrogen affects the expression of neurofilament proteins, microtubulin-associated proteins, TAU and GAP43 (Scoville et al., 1997). In addition to neurotrophic factors, structural proteins and anti-apoptotic factors; thiol antioxidants and their related enzymes, such as thiol disulfide oxido-reductases including Grx1 may represent a fourth class genes that are regulated by estrogen in the CNS and are involved in neuroprotection particularly those related to mitochondrial dysfunction.

The studies described herein suggest that estrogen protects against mitochondrial dysfunction by maintaining cellular redox status through higher constitutive expression of thiol disulphide oxido-reductase such as, glutaredoxin in the CNS (Fig. 6). Up regulation of glutaredoxin, *in vivo* may offer a therapeutic target for neuroprotection against excitotoxicity in motor neurons.

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