Glutaredoxin is essential for maintenance of brain mitochondrial complex I: studies with MPTP[1]

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SPECIFIC AIMS

Mitochondrial complex I dysfunction has been associated with neurodegenerative disorders such as Parkinson's disease. As complex I is known to have essential thiol groups in its subunits, we examined the role of glutaredoxin, a thiol disulfide oxidoreductase (also known as thioltransferase), in maintaining the functional integrity of mitochondrial complex I in the brain under normal conditions and following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that produces Parkinson's disease-like symptoms in primates, including humans through inhibition of complex I.

PRINCIPAL FINDINGS

1. Down-regulation of glutaredoxin expression leads to inhibition of mitochondrial complex I activity

Glutaredoxin was down-regulated selectively in the brain of Swiss albino mice by injecting phosphorothionate end-capped sense and antisense oligonucleotides to glutaredoxin (400 μg in 2 divided doses at a 12-h interval), intrathecally. Selective down-regulation of glutaredoxin in the frontal cortex and striatum resulted in significant loss of complex I activity in the frontal cortex and striatum as compared with corresponding controls ($P<0.005$), and animals treated with vehicle or sense nucleotides were unaffected (**Fig. 1*a***, ***b***, and **Fig. 2**). In the hippocampus, glutaredoxin was not down-regulated, and complex I activity was similar to vehicle-treated controls (Fig. 2). These results reveal the important role of glutaredoxin in maintaining functional integrity of mitochondrial complex I in brain regions under normal conditions.

2. Mitochondrial complex I inhibition caused by down-regulation of glutaredoxin expression is a result of impaired thiol status of the enzyme

Complex I activity was measured after incubating mitochondria from antisense oligonucleotide-treated animals with the thiol reductant DTT; this completely restored complex I activity to the levels similar to corresponding controls (Fig. 1*c*), indicating that the oxidation of critical thiol groups in the enzyme complex was responsible for the loss of enzyme activity caused by down-regulation of glutaredoxin.

3. Up-regulation of glutaredoxin is essential for recovery of mitochondrial complex I function from toxic insult caused by MPTP administration

We examined the relationship between the functional status of glutaredoxin and complex I in the striatum of mice following a single, systemic dose of MPTP (30 mg/kg body weight, s.c.). Complex I activity was significantly decreased at 0.5 h after MPTP administration. However, after 4 h, complex I activity recovered and was significantly higher than corresponding controls (121% of controls; $P<0.002$). During the recovery of complex I activity in the striatum glutaredoxin, activity increased significantly to 190% of the corresponding controls ($P<0.002$). However, glutaredoxin activity was similar to controls 18 h after MPTP administration, and simultaneously, loss in complex I activity was observed. Thus, a direct correlation was seen between the functional status of complex I and glutaredoxin activity.

4. Glutaredoxin protein expression increased in the striatum following a single dose of MPTP administration

To examine whether the increase in glutaredoxin activity in the striatum was a result of increased expression of glutaredoxin protein, immunoblot and immunohistochemical analyses were performed using antibody to human red blood cell glutaredoxin, which shares 100% homology with the brain glutaredoxin. Immunoblot analysis showed a significant increase in glutaredoxin protein expression 4 h after MPTP administration in the striatum compared with corresponding controls ($P<0.05$). In the hippocampus, a brain region unaffected by MPTP, no change was observed in the expression levels of glutaredoxin. Immunohistochemical analysis revealed increased expression of glutare-

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.-02–0771fje; to cite this article, use *FASEB J.* (February 19, 2003) 10.1096/fj.02–0771fje

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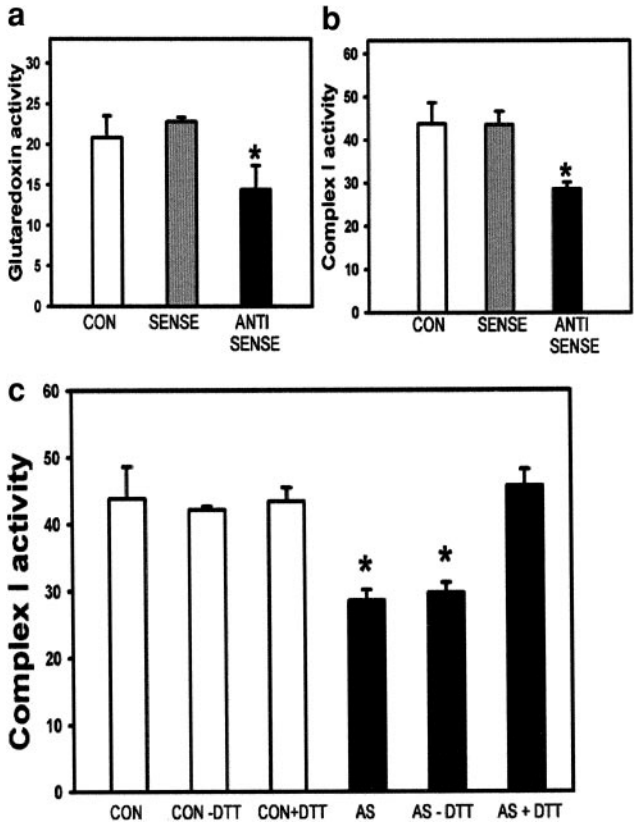

Figure 1. Disulfide reductant dithiothreitol (DTT) restores mitochondrial complex I activity lost by down-regulation of glutaredoxin. Mice were administered sense or antisense (AS) oligonucleotides to glutaredoxin in two divided doses (200 μg/dose, intrathecally) at 12-h intervals, the animals were killed 12 h after the last injection, and the striatum was dissected out. Control (CON) animals received vehicle alone. *a)* Glutaredoxin activity was measured in the striatum. Complex I activity was measured in the mitochondria of the striatum in the absence (*b*) and presence (*c*) of DTT. Complex I activity is expressed as nmole of reduced nicotinamide adenine dinucleotide (NADH) oxidized/min/mg of protein, and glutaredoxin activity is expressed as nmole of NADH phosphate (NADPH) oxidized/min/mg of protein. Values are mean ± SD (n=3 animals). *, Values are significantly different from control animals (P<0.001).

doxin protein in striatal neurons of MPTP-treated animals compared with vehicle-treated animals.

5. Glutaredoxin mRNA expression increased in the striatum following MPTP administration

Total RNA was extracted from the striatum of control and MPTP-treated mice, and Northern blot analysis was preformed using cDNA to glutaredoxin. We observed an increased level of glutaredoxin mRNA 1 h after MPTP administration in the striatum compared with vehicle-treated animals. In situ hybridization studies also revealed increased transcription of glutaredoxin mRNA in striatal neurons of MPTP-treated animals compared with vehicle-treated animals.

6. Redox-sensitive transcription factors, activated protein 1 (AP1), antioxidant response element (ARE), and nuclear factor (NF)[κ]B, are activated in the striatum following a single dose of MPTP administration

Nuclear extracts were prepared from the striatum of vehicle, and MPTP-treated mice, electrophoretic mobility shift assays (EMSAs), and supershift assays were performed using the nuclear extracts and ^{32}P-labeled oligonucleotides containing consensus-binding sequences for AP1, ARE, or NF[κ]B. EMSA revealed significantly increased binding of AP1, ARE, and NF[κ]B within 30 min of MPTP administration compared with corresponding

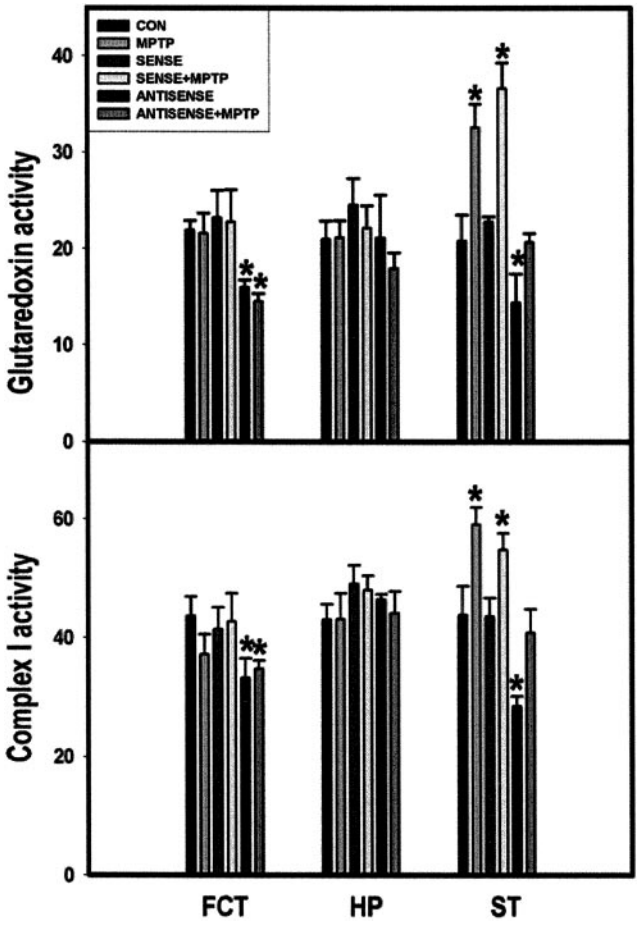

Figure 2. Effect of down-regulation of glutaredoxin expression on MPTP-mediated mitochondrial complex I activity. Mice were administered sense or antisense oligonucleotides to glutaredoxin in two divided doses (200 μg/dose, intrathecally) at 12-h intervals. MPTP [30 mg/kg body weight, subcutaneously (s.c.)] was injected 8 h after the second injection of oligonucleotides. Animals were killed 4 h after MPTP administration. Another group of animals were administered MPTP alone and were killed 4 h later. Control animals received vehicle alone. Glutaredoxin and complex I activities were measured in the frontal cortex (FCT), hippocampus (HP), and striatum (ST). Complex I and glutaredoxin activities are expressed as nmoles of NADH oxidized/min/mg of protein and nmoles of NADPH oxidized/min/mg of protein, respectively. Values are mean ± SD (n=3 animals). *, Values significantly different from vehicle-treated controls (P<0.001).

controls ($P<0.005$). The activation of AP1 was supershifted by antibody to *p*-c-Jun, JunB, and c-Fos, indicating the possible involvement of these proteins in the activation of the AP1 complex.

Thus, 30 min after MPTP administration, we observed activation of the AP1 transcription factor followed by increased transcription of glutaredoxin mRNA at 1 h Increased expression of glutaredoxin protein and activity was seen at 4 h, which in turn mediated recovery of complex I function caused by MPTP administration.

7. Glutaredoxin is critical during recovery of mitochondrial complex I function from toxic insult caused by MPTP administration

The role of glutaredoxin in mediating recovery of complex I function following MPTP administration (30 mg/kg body weight, s.c.) was further confirmed by administration of MPTP to mice pretreated with antisense oligonucleotides to glutaredoxin. Antisense oligonucleotides were administered intrathecally to mice at a dose of 200 μg twice at a 12-h interval. MPTP was administered 8 h after the second injection of the oligonucleotides. The frontal cortex, hippocampus, and striatum were dissected out, and glutaredoxin and complex I activities were assayed. Antisense oligonucleotides completely abolished the recovery of complex I in the striatum, which would otherwise have occurred 4 h after MPTP administration, demonstrating the critical role played by glutaredoxin in restoring complex I function (Fig. 2).

CONCLUSION AND SIGNIFICANCE

Complex I is known to possess active thiol groups and is extremely sensitive to the thiol status in cells. Depletion of reduced glutathione (GSH) in PC12 cells through down-regulation of γ-glutamyl cysteine synthetase (the rate-limiting enzyme in GSH synthesis) leads to selective inhibition of complex I. However, the factors involved in maintenance of complex I under normal conditions and during recovery from oxidative stress are not well defined.

This study demonstrates the critical role of glutaredoxin in maintaining the functional integrity of brain mitochondrial complex I under normal conditions and during recovery from oxidative stress such as that mediated by MPTP, a neurotoxin that causes Parkinson's disease. Complex I dysfunction has been noted in post-mortem brain samples and peripheral tissues from Parkinson's disease patients; however, the mechanisms underlying this dysfunction remain unclear (**Fig. 3**). Loss of GSH is also seen in the substantia nigra of patients with Parkinson's disease. Inhibition of glutathione synthesis has been shown to selectively result in complex I loss in PC12 cell lines.

Glutaredoxin, a thiol disulfide oxidoreductase discovered 25 years ago, reduces protein glutathione-mixed disulfides to protein thiols using reducing equivalents of GSH. Although the potential role of glutaredoxin in protection against oxidative injury has been hypothesized, its regulation during oxidative stress has not been clearly defined through experimental evidence. The present study provides evidence of differential regulation of glutaredoxin at the transcriptional level during recovery of mitochondria from oxidative stress and also provides evidence for its role in maintaining functionality of mitochondrial complex I in normal conditions. This is of significance, as maintenance of reduced thiol status of mitochondrial complex I is important not only for proper mitochondrial function but also in the management of neurodegenerative diseases, such as Parkinson's disease, which are associated with complex I dysfunction.

This study provides irrevocable evidence for the role of glutaredoxin in maintaining complex I function and thus ensuring mitochondrial integrity. Further, glutaredoxin plays a critical role in the recovery of complex I impaired by oxidative stress caused by neurotoxins such as MPTP. FJ

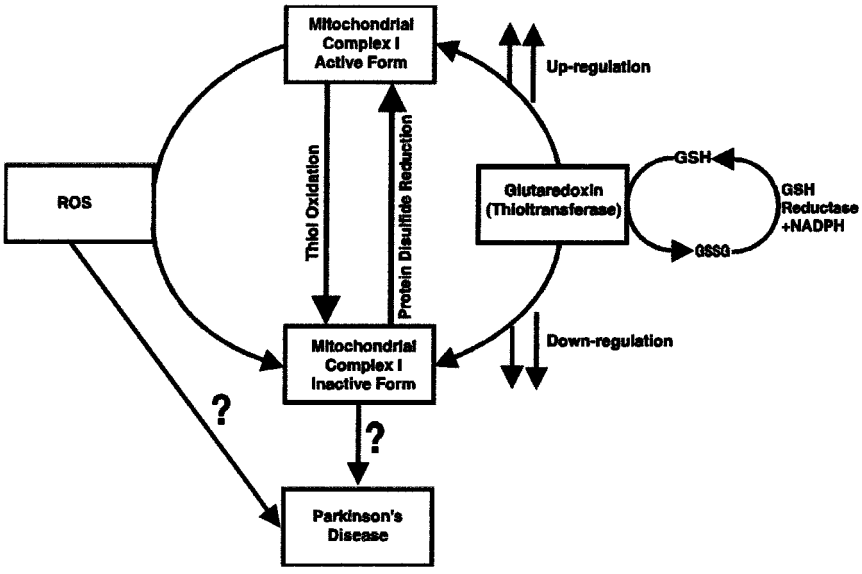

Figure 3. Schematic illustration depicting the important role of glutaredoxin (a thiol disulfide oxido-reductase) in maintaining functional integrity of mitochondrial complex I under normal conditions and during oxidative stress caused by generation of reactive oxygen species (ROS). Reduced protein thiol status is essential for complex I function and down-regulation of glutaredoxin is associated with loss of complex I activity. Oxidative stress which could lead to protein thiol oxidation and complex I dysfunction has been associated with Parkinson's disease.