

Protein Levels and Activity of Some Antioxidant Enzymes in Hippocampus of Subjects with Amnesic Mild Cognitive Impairment

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Abstract Mild cognitive impairment (MCI) is generally referred to the transitional zone between normal cognitive aging and early dementia or clinically probable Alzheimer's disease (AD). Most individuals with amnesic MCI eventually develop AD, which suggests that MCI may be the earliest phase of AD. Oxidative stress is observed in brain from subjects with both AD and MCI. Among others, two possibilities for elevated oxidative stress are decreased activity or elevated expression of antioxidant enzymes, the latter as a response to the former. Accordingly, in the current study, the protein levels and activity of some antioxidant enzymes in the hippocampus of control and MCI brain were measured using Western blot analysis and spectrophotometric methods, respectively. Alterations in the levels and activity of a number of antioxidant enzymes in MCI brain compared to age-matched controls were found. These results are consistent with the hypothesis that oxidative stress may be an early event in the progression of amnesic MCI to AD.

Keywords Mild cognitive impairment · Oxidative stress · Hippocampus · Glutathione · Glucose-6-phosphate dehydrogenase · Glutathione reductase · Glutathione peroxidase · Superoxide dismutase

Introduction

Mild cognitive impairment (MCI) is generally referred to the transitional zone between normal cognitive aging and early dementia or clinically probable Alzheimer's disease (AD). MCI is classified into two major types: amnesic MCI and nonamnesic MCI. Most individuals with amnesic MCI were reported to eventually develop AD, which suggests that MCI may be the earliest phase of AD [1–4]. A number of studies showed elevated levels of oxidative stress markers in MCI brain, indexed by higher levels of protein, lipid, carbohydrate, RNA, and DNA oxidation [5–9]. Increased production of free radicals in the MCI brain likely may be an essential factor in the pathogenesis and progression of AD and, consequently, is a potential target of therapeutic strategies.

Oxidative stress has been shown to be associated with a number of neurological diseases such as AD, Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS), among others. Oxidative stress is the condition arising from imbalance between toxic reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, and antioxidant systems. Oxidative stress also could occur due to a decrease in the levels of antioxidants without much change in the levels of reactive oxygen species. Different tissues have different susceptibilities to oxidative stress. Brain is particularly more vulnerable to oxidative damage due to relatively low levels of antioxidants, high levels of polyunsaturated fatty acids (which are facile

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targets of free radical attack), high level of oxygen utilization, and an inherently high flux of ROS generated during neurochemical reactions such as dopamine oxidation [10, 11].

One of the antioxidants found predominantly in the brain is glutathione (GSH), a non-protein thiol present in millimolar concentrations [12]. GSH can protect cells against ROS and also can protect cells against the toxic effects of lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE). This GSH-mediated protection leads to GSH oxidation to form glutathione disulfide (GSSG), a reaction catalyzed by glutathione peroxidase (GPx). Recycling of GSSG back to GSH is catalyzed by glutathione reductase (GR), which uses NADPH as a cofactor [12]. In addition to its antioxidant property, GSH also is involved in biosynthetic processes such as enzyme activity regulation, DNA repair, and activation of transcription factors [13]. Hence, an imbalance in the levels of GSH and GSSG could be detrimental to cell survival, and the ratio of GSH:GSSG has been used as an index of oxidative stress [14]. A decrease in the levels of reduced glutathione has been associated with aging and many diseases including AD [15, 16].

Another cellular defense enzyme is glutathione *S*-transferases (GST), a group of detoxification enzymes, that catalyze glutathione conjugation with nucleophilic compounds such as HNE and acrolein, products of lipid peroxidation [17], and the resulting glutathione-*S*-conjugates are effluxed from cells by the multidrug resistant protein-1 (MRP-1), an ATP binding cassette (ABC) family protein [18, 19]. Studies also have shown reduced GST activity and increased expression in AD brain that correlated well with the HNE-induced modification of GST [20]. In addition, an *in vitro* study using neuronal cell culture showed that increased expression GST protected cells against oxidative stress induced by HNE [20].

In the present study the protein levels and activities of GR, GPx, and SOD were measured in control and MCI hippocampus, a region of the brain that has been reported to be severely affected in AD. In addition, the activity of GST, glucose-6-phosphate dehydrogenase (G6PDH), and the ratio of GSH and GSSG were determined. Alterations in the antioxidant system in the MCI hippocampus compared to that of control suggest that oxidative imbalance and subsequent oxidative stress are early events in the progression of MCI to AD.

Materials and Methods

Control and MCI Brains

Frozen hippocampus samples were obtained from six MCI patients and six age-matched controls for the present study.

The Rapid Autopsy Program of the University of Kentucky Alzheimer's Disease Clinical Center (UK ADC), provided the specimens, whose post mortem intervals were extremely short: 2.9 ± 0.5 h for controls and 3.1 ± 0.4 h for MCI. The brain tissues (hippocampus) from control or MCI were homogenized in a lysis buffer (10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 , 0.6 mM MgSO_4) containing the protease inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml), trypsin inhibitor (0.5 mg/ml), and PMSF (40 mg/ml). Homogenates were centrifuged at $15,800 \times g$ for 10 min to remove debris. The supernatant was used to determine the total protein concentration by the BCA method (Pierce, Rockford, IL).

Western Blot Analysis

Immunoblot analysis was performed with the supernatant obtained from hippocampus from control and MCI. Briefly, 75 μg of the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the proteins to nitrocellulose membranes at 160 mA/gel for 2 h. The nitrocellulose membranes were blocked for 1 h at room temperature in fresh blocking buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4, containing 5% BSA). This procedure was then followed by incubation of the membrane with primary antibodies against GR, GPx, or MnSOD (1:1000) made in wash blot [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, pH 7.4] with 3% BSA. Following three washes with wash blot, the blots were incubated with secondary antibody conjugated with alkaline phosphatase in wash blot for 1 h at room temperature. The blots were washed again three times in wash blot, and the bands were visualized using Sigma fast tablets (5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) and Nitro Blue Tetrazolium (NBT)) as substrates. Blots were dried, scanned with Adobe Photoshop (San Jose, CA), and quantitated with Scion Image.

GSH Assay

Samples were deproteinated by treatment with 10% (w/v) metaphosphoric acid (Aldrich, Milwaukee, WI) and centrifuged at 2,000 g for 2 min. The supernatant was collected and treated with 4 M triethanolamine (TEAM) solution. After TEAM treatment, the GSH and GSSG levels were determined using a GSH assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's directions.

GR Activity

The assay system to estimate GR (EC 1.6.4.2) activity consisted of 0.1 M phosphate buffer (pH 7.6), 0.5 mM

EDTA, 1.0 mM oxidized glutathione, 0.1 mM NADPH and 10 μ l PMS in a total volume of 200 μ l [21]. The enzyme activity was assayed in a 96-well plate reader by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein.

G6PDH Activity

G6PDH was measured using a standard assay mixture [Tris-HCl buffer (0.05 M) pH 7.6, NADP⁺ (0.1 mM), MgCl₂ (8 mM) and glucose-6-phosphate (0.8 mM)]. The reaction was monitored in a 96-well plate reader by measuring the increase of absorbance at 340 nm.

GST Activity

GST activity was measured essentially as described by Habig and Jakoby [22] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The standard assay mixture contained 1 mM CDNB, 1 mM GSH and 100 mM potassium phosphate buffer (pH 6.5) in a volume of 1 ml. The reaction was followed at 340 nm.

SOD Activity

SOD total was measured using a buffer (G buffer) that contained 0.05 M glycine, 0.1 M NaOH and 0.1 M NaCl, pH 10.3 and epinephrine. The reaction was monitored in a

96-well plate reader by measuring the decrease of absorbance at 480 nm.

Statistical Analysis

The results are presented as means \pm SD. Statistical evaluation was performed using a Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

Results

The expression and activity of various antioxidant enzymes were determined in control and MCI hippocampus. Western blot analyses of hippocampus samples probed with anti-MnSOD and anti-GR antibodies (Figs. 1 and 2, respectively) showed a significant increase in the expression of these proteins in MCI brain compared to control brain ($P < 0.05$). SOD activity was significantly decreased in MCI hippocampus compared to the control ($P < 0.05$). No difference in activity of GR was found in MCI hippocampus compared to control.

No significant difference was observed in the protein levels or activity of GPx between MCI and controls (Fig. 3). G6PDH activity (Fig. 4) was significantly elevated ($P < 0.05$) in MCI hippocampus compared to that of control. In contrast, glutathione-*S*-transferase activity was significantly decreased ($P < 0.05$) in MCI samples, compared to control (Fig. 5). The ratio of GSH/GSSG was slightly, but significantly, decreased in MCI hippocampus (Fig. 6).

Fig. 1 Superoxide dismutase levels and activity in control and MCI brain. (a) Western blot showing the levels of superoxide dismutase in control and MCI hippocampus (HP). (b) represents histogram. (c) shows superoxide dismutase activity. See text for methods employed. $n = 6$, $*P < 0.05$

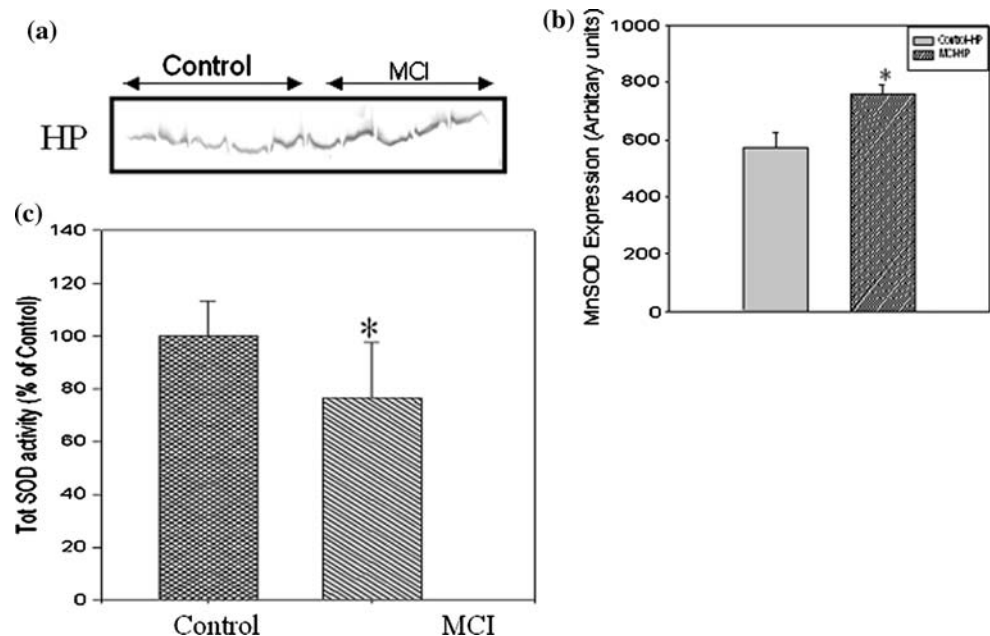


Fig. 2 Glutathione reductase levels and activity in control and MCI brain. (a) Western blot showing the levels of glutathione reductase in control and MCI and hippocampus (HP). (b) represent histogram. (c) shows GR activity. See text for methods employed. $n = 6$, $*P < 0.05$

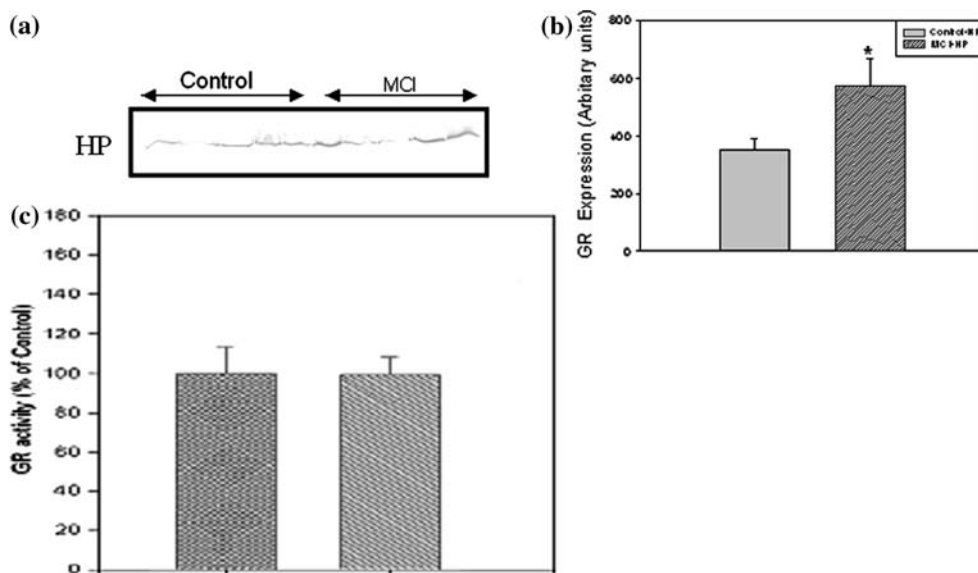


Fig. 3 Glutathione peroxidase levels and activity in control and MCI brain. (a) Western blot showing the levels of glutathione peroxidase in control and MCI hippocampus (HP). (b) represents histogram. (c) shows GR activity. See text for methods employed. $n = 6$, $*P < 0.05$

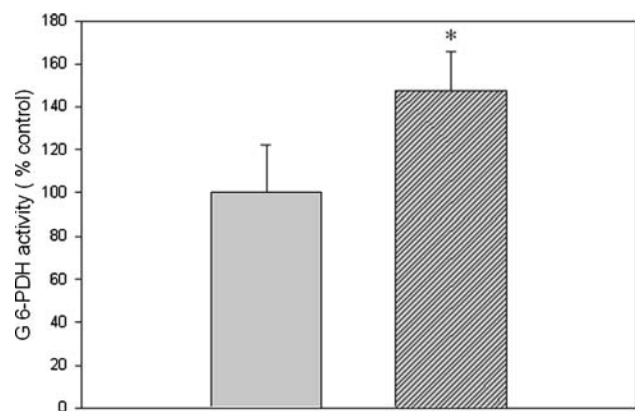
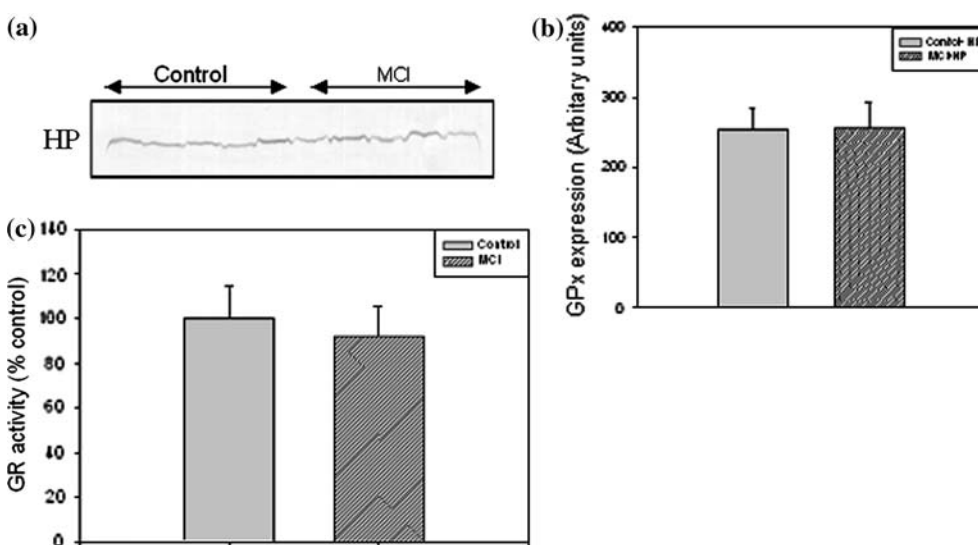


Fig. 4 The activity of glucose 6-phosphate in control and MCI brain. See text for methods employed. $n = 6$, $*P < 0.05$

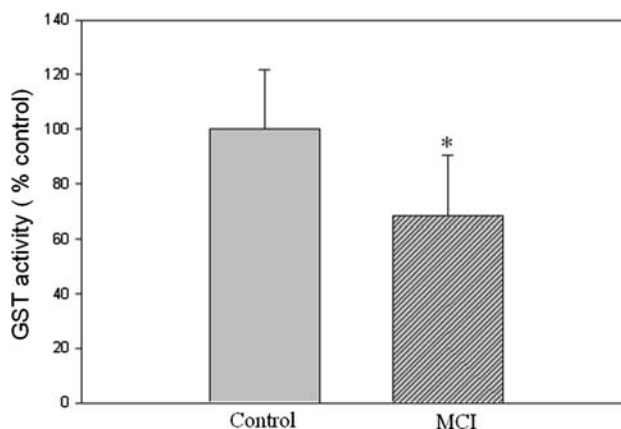


Fig. 5 The activity of glutathione S-transferases in control and MCI brain. See text for methods employed. $n = 6$, $*P < 0.05$

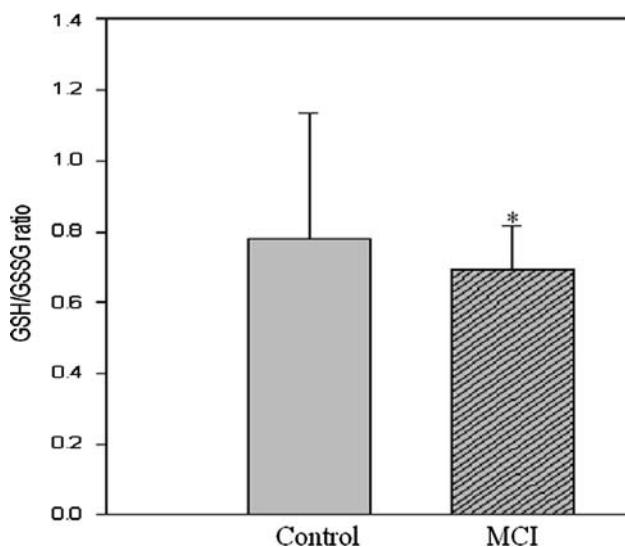


Fig. 6 Ratio of reduced to oxidized glutathione in control and MCI brain. See text for methods employed. $n = 6$, $*P < 0.05$

Discussion

Amnesic MCI involves loss of memory and histologically shows the loss of brain cells, and about 70% of MCI cases were reported to eventually transform into AD [2, 3]. The cause of AD is not yet clearly understood, but a number of hypotheses are proposed to explain AD pathology, one of which is elevated oxidative stress. Indeed, elevated oxidative stress is found in AD [23] and MCI brain [6–9]. Further, a number of oxidatively modified proteins have been reported in MCI brain that correlated with the pathology of MCI [5, 23, 24]. Since oxidative stress involves an imbalance in the generation of ROS and the antioxidant system, in the present study we measured the protein levels and the activity of some components of the antioxidant systems in hippocampus, a region of the brain that is severely affected in AD. We observed alterations in activities and protein levels of some of the antioxidant enzymes in MCI brain consistent with the observed oxidative stress in MCI [5–9] and suggesting that an antioxidant imbalance may play a role in the progression of amnesic MCI to AD. GSH plays a fundamental role in maintaining the reduced status in the cell. The data obtained from the current study showed a significant decrease in the GSH/GSSG ratio in MCI hippocampus compared to controls (Fig. 6) suggesting a more oxidized redox state of the cell that may eventually lead to elevated oxidative stress. In AD brain, the ratio of GSH and GSSG has been reported to be altered as indicated by decreased levels of GSH and increased levels of GSSG in specific regions. The decreased levels of GSH in AD brain possibly contribute to the oxidative stress observed in AD that may eventually lead to neuronal cell dysfunction and neuronal

loss [12, 25]. The ratio of GSH and GSSG is regulated in the cells by two main enzymes, i.e., GPx and GR. GPx detoxifies peroxides with GSH acting as an electron donor in the reduction reaction, producing GSSG as an end product. The reduction of GSSG to GSH is catalyzed by GR in a process that requires NADPH, and NADPH is derived from the pentose phosphate pathway [26] in which G6PDH participates with considerable importance, catalyzing NADPH production [27]. Hence, GR plays an important role by assuring a restoration of the reduced glutathione pool, which is fundamental to maintain the reduced state of cells [28, 29].

In this current study, the activity and the protein levels of GPx did not show any significant increase in MCI brain regions, whereas we observed a significant increase in the GR expression with no change in its activity that reduces GSSG. The functional impairment of GR could be due to its oxidative modification, as exemplified by some of the proteins identified by redox proteomics in AD and MCI brain from our laboratory [18, 24]. Further, G6PDH activity was observed to be significantly increased in MCI hippocampus compared to control. This increase in G6PDH activity could be in response to oxidative stress in MCI hippocampus with the result that more NADPH is produced. However, this could also have a negative impact as discussed below.

SOD catalyzes the conversion of the one electron reduced species of molecular oxygen to hydrogen peroxide and oxygen [30, 31]. At physiological conditions superoxide radicals are produced mainly from the respiratory chain, but they can also be synthesized by activated microglia [29]. Further, an increased level of NADPH, as a consequence of increased levels of G6PDH also may trigger the activity of NADPH oxidase, another source of superoxide [32]. In addition, the current study revealed that activity of SOD is decreased, which could be related to increased lipid peroxidation, protein carbonylation and nitration, and DNA/RNA oxidation in MCI brain [6–9]. In MCI brain, the level of SOD is elevated, while the total SOD activity is decreased (Fig. 1). We speculate that the elevated levels of MnSOD are in response to lower activity or to oxidative modification of this enzyme. However, a contribution of Cu,Zn-SOD expression and activity to the total SOD activity can not be excluded.

Though the current study did not reveal a large reduction in the GSH:GSSG ratio in MCI brain, a decrease in the activity of GST, a detoxification enzyme that requires GSH for its functions, was observed. This decrease in the activity of GST may be due to its oxidative modification. Indeed, significantly decreased activity of GST in AD brain correlated well with HNE modification of this enzyme, a modification that may lead to decreased removal of electrophilic compounds such as HNE from neurons, which

might trigger cell death [18, 20]. In addition, MRP-1 has been demonstrated to have HNE covalently bound to it that presumably impairs clearance of GS-HNE conjugates from the cells [18]. A number of previous studies showed that, generally, oxidative modification of a protein leads to the functional impairment of the protein [5, 23, 24, 33–37]. This impairment of protein function may play a role in promoting neuronal cell death.

A previous study in MCI and AD patients showed decreased levels of plasma non-enzymatic antioxidants and the activity of antioxidant enzymes [38]. These findings suggest that increased free radical production in MCI might lead to a rapid consumption of plasma antioxidants. In a vicious cycle, MCI and subsequently AD individuals may have an inadequate antioxidant enzymatic activity that might account for increased free radical production. Conceivably, this may be one of the mechanisms involved in the progression of MCI to AD.

One of the approaches to delay the onset or the progression of MCI to AD may be to augment antioxidant defense systems either by dietary or pharmacological intake of antioxidants [39–41]. There are a number of free radical scavengers, such as vitamins E and C, flavonoids, *Ginkgo biloba* extract EGb 761, and carotenoids etc., that at reasonable concentrations have no major side effects.

In conclusion, the alterations in the expression and activity of some of the antioxidant enzymes in MCI hippocampus may be considered an adaptive response to dynamically regulate cellular defence mechanisms against oxidative stress. Our results underscore the role of the antioxidant system against increased oxidative stress in MCI brain and are consistent with the notion of a direct correlation between elevated radical species and progression of amnesic MCI to AD.

Final Comments

This paper is being published in a special issue of *Neurochemical Research* in honor of Professoressa Anna-Maria Giuffrida Stella of the University of Catania. Professor Butterfield has known and admired the research of Professoressa Giuffrida Stella for many years. Her extraordinary and pioneering contributions to the discipline of neurochemistry are numerous and important, both in the laboratory and for the International Society for Neurochemistry. Professor Butterfield has given lectures at the University of Catania many times, and each time he visits Sicily, Professoressa Giuffrida Stella is unfailingly gracious in her hospitality. Professor Butterfield is proud to call Professoressa Giuffrida Stella a good friend and colleague, and he wishes her continued success in neurochemistry as she enters retirement and remains active in research.

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