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## Quantification of antioxidant activity in brain tissue homogenates using the ‘total equivalent antioxidant capacity’

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### Abstract

We demonstrate herein that the standard ‘Trolox equivalent antioxidant capacity’ (TEAC) assay, typically utilized to quantify total antioxidant levels within plasma, can also be utilized for tissue homogenates. Normal mice and transgenic mice lacking apolipoprotein E were subjected to a diet including iron as a generic pro-oxidant for 1 month (which has been shown to induce oxidative damage in our prior studies) and homogenates of brain tissue were subjected to the TEAC assay. Levels of the endogenous antioxidant glutathione levels were also monitored by HPLC. As described previously, ApoE-deficient mice expressed increased levels of glutathione; total antioxidant levels, as determined by TEAC, were increased to a similar extent. The increase in total antioxidant levels, as determined by TEAC, following dietary iron challenge paralleled the increase in glutathione levels, as determined by HPLC. These findings indicate that the TEAC assay may be useful for tissue homogenates. The rapid nature of this assay compared to HPLC, coupled with its lack of requirement for sophisticated equipment, makes it well suited for analyses of multiple tissue samples.

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### 1. Introduction

Oxidative stress is a pivotal factor in chronic neurodegenerative conditions, such as Alzheimer’s disease (AD) (Nourhashemi et al., 2000; Smith et al., 2000). Oxidative damage initiates at the membrane, encompasses cytoskeletal protein and nucleic acid damage, and ultimately extends to regions beyond the amyloid- $\beta$  lesions themselves, indicating the presence of generalized oxidative damage (Yan et al., 1994; Nourhashemi et al., 2000; Smith et al., 2000). Restoring or maintaining oxidative buffering capacity therefore represents one

useful therapeutic approach to minimize neurodegeneration.

Deficiency in dietary antioxidants or precursors of antioxidants enhanced oxidative damage (Reich et al., 2001; Shea and Rogers, 2002). Experimental elevations in the endogenous antioxidant glutathione and in dietary antioxidants in AD brain were capable of reducing oxidative damage and delaying progression of the disease (Sano et al., 1997; Ramassamy et al., 1999; Pocernich et al., 2001). Nevertheless, oxidative stress can in some instances overwhelm the buffering capacity of endogenous glutathione (Lievre et al., 2001).

Certain phenotypes that place an individual at risk for AD can be accompanied by compensatory increases in endogenous antioxidants (Ramassamy et al., 2002). The epsilon 4 allele of the apolipoprotein E gene (ApoE) is linked with an increase in and an earlier age of onset of

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AD (Growdon, 2001). The extent of oxidative damage in the brains of AD patients correlates with the presence of the E4 allele of ApoE (ApoE4) (Ramassamy et al., 1999). Increased levels of glutathione and increased activities of enzymes that participate in its formation, such as glutathione peroxidase, were observed in AD cases bearing ApoE4 (Ramassamy et al., 1999, 2002). Transgenic mice lacking ApoE are useful models for examining the impact of lack of this protein on brain tissue, as well as for developing therapeutic approaches. Similar to AD patients bearing the ApoE4 allele, ApoE-deficient mice demonstrate increased susceptibility to lipid peroxidation under conditions that promote oxidative stress (Hayek et al., 1994; Shea and Rogers, 2002; Shea et al., 2002a,b). Also similar to AD patients bearing the ApoE4 allele, ApoE-deficient mice exhibit increased levels of glutathione and activities of enzymes that form it, in brain tissue, which may represent an attempt to compensate for increased reactive oxygen species due to diminished ApoE function (Huang et al., 2000; Shea et al., 2002a). Increased dietary antioxidants and antioxidant precursors alleviate the deleterious impact of ApoE deficiency in these mice (Shea and Rogers, 2002; Shea et al., 2002a,b; Veinbergs et al., 2000).

A simple method to quantify overall oxidative buffering capacity in brain tissue would be useful to monitor the efficacy of therapeutic approaches towards maintaining antioxidant levels in AD and in experimental models of AD. We present data herein that the ‘Trolox equivalent antioxidant capacity (TEAC)’ assay, typically utilized to quantify antioxidant levels in plasma using Trolox (a water-soluble form of vitamin E) as a standard (Miller et al., 1993), can also be applied to brain tissue homogenates. As a control for the accuracy of the TEAC assay, we also quantified levels of the endogenous antioxidant glutathione by previously-demonstrated HPLC methodologies (Shea et al., 2002a), since glutathione levels are increased under conditions of oxidative stress (Miller et al., 1993) and, as stated above, glutathione is reportedly present at higher levels in ApoE-deficient mice versus normal mice.

## 2. Methods

Normal C57Bl/6J mice and ApoE<sup>tm1Une</sup> homozygous ‘knockout’ mice on a C57Bl/6J background (Jackson Laboratories, Bar Harbor, ME) were maintained for 1 month on a basal diet (‘AIN-76’; Purina; Whittaker et al., 1996; Ibrahim et al., 1997) with and without a mixture of fish and corn oil (8 and 2% of the total diet wet weight, respectively) and iron (as ferric citrate; 4/500 g total wet weight of AIN-76 basal diet mixture; diet and water ad libitum) as described previously (Shea and Rogers, 2002; Shea et al., 2002b). While we utilized

dietary iron as a ‘generic’ pro-oxidant (Whittaker et al., 1996; Ibrahim et al., 1997), it has been speculated that iron may be a contributing factor in AD (Smith and Perry, 1995). Total brain tissue was harvested and frozen at  $-80^{\circ}\text{C}$  until use.

Samples of brain tissue were homogenized (50 strokes in a glass–glass homogenizer) in 1.55 M KCl in 0.05 M phosphate-buffered saline (pH 7.4) at a final concentration of 200 g protein/l of buffer, then sonicated for  $2 \times 5$  s (40 W output) with a Branson sonifier with microtip. Total antioxidant capacity was quantified according to Miller et al. (1993). Homogenates were diluted with 1.5 vol of phosphate-buffered saline containing 2,2-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) and the chromogenic peroxidase metmyoglobin in the presence of hydrogen peroxide. This generates the radical cation ABTS<sup>+</sup> and resultant absorbance that was recorded at a single time point at 270 s. A reagent/diluent blank reading was subtracted from the absorbances at that point. The sample concentration was then interpolated from a slope average curve derived from a calibrator that was concurrently run (Miller et al., 1993). The degree of quenching of radical generation in individual samples, indicative of the presence of antioxidant activity, was quantified by comparison with a standard curve of 1 mM Trolox and expressed as ‘Trolox equivalent antioxidant capacity’ or TEAC. Samples were derived from three to four normal and three to four ApoE<sup>-/-</sup> mice for each dietary condition, from two independent experiments (total  $n = 6$  for each diet for both experiments).

Glutathione levels were quantified in brain homogenates according to Araki and Sako (1987) with modifications. Homogenates (100  $\mu\text{l}$ ) were reduced by mixing with 50  $\mu\text{l}$  of 100  $\mu\text{M}$  *N*-acetylcysteine (as an internal standard) and 10  $\mu\text{l}$  of tri-carboxyethylphosphine (100 mg/ml in 0.05 M HCl). Samples were vortexed, incubated at room temperature for 30 min, then centrifuged at  $10\,000 \times g$  for 10 min. Some 80  $\mu\text{l}$  of the resulting supernatant were combined with 160  $\mu\text{l}$  of 2 M boric acid/4 mM EDTA (pH 10.5), followed by 80  $\mu\text{l}$  of 1.0 mg/ml SBDF [7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate] in boric acid/EDTA buffer. Samples were mixed, incubated for 60 min at  $60^{\circ}\text{C}$ , equilibrated to reach temperature and 50  $\mu\text{l}$  injected into a Hewlett Packard model 1090 HPLC used with a 1046A fluorescence detector and a Hewlett Packard  $4.6 \times 60$  mm high-speed analytical column packed with 3  $\mu\text{M}$  ODs (C18) Hypersil silica. The mobile phase consisted of methanol/0.2 M acetate buffer pH 4.0 (2/98 by volume). Glutathione concentrations were then determined by comparison of peak area ratios of glutathione to the internal standard *N*-acetylcysteine in the sample compared to peak area ratios obtained for standards. Samples were derived from three to four normal and three to four ApoE<sup>-/-</sup> mice for each dietary condition, from two or

more independent experiments (total  $n = 6$  for each diet for both experiments).

### 3. Results and discussion

Brain tissue homogenates from ApoE-deficient mice exhibited a significantly ( $P \leq 0.005$ ; Student's *t*-test) increased oxidative buffering capacity versus homogenates from normal mice (Fig. 1A), indicating that the lack of ApoE activity fostered an increase in one or more endogenous antioxidants. Challenge with dietary iron further increased oxidative buffering capacity in both normal and ApoE mice, indicating endogenous antioxidants were upregulated in brain tissue of both mice in response to oxidative stress. These changes in total antioxidant buffering capacity were mirrored by glutathione levels in brain tissue. Consistent with prior studies (Hayek et al., 1994), brain tissue of ApoE-deficient mice demonstrated an  $\approx 20\%$  increase in glutathione levels over those in normal mice ( $P \leq 0.005$ ; Student's *t*-test; Fig. 1B). Dietary iron increased glutathione levels in brain tissue of both normal and ApoE-deficient mice. Notably, however, ApoE-deficient mice displayed a marked further increase than the CNS of normal mice under all conditions tested (Fig. 1B). Notably, no statistical difference was observed in comparing oxidative buffering capacity in the presence versus absence of iron in normal mice nor in ApoE-deficient mice ( $P \geq 0.05$ ), highlighting the potentially deleterious combination of genetic deficiency and dietary oxidative stress (Shea and Rogers, 2002; Shea et al., 2002a,b). By contrast, specific analyses of glutathione

levels did reveal a significant difference when comparing values obtained for normal mice with versus without dietary iron and for ApoE-deficient mice with versus without dietary iron.

The similarity in changes in total antioxidant capacity under various diets as quantified by the TEAC assay with that of the identified endogenous glutathione as quantified by HPLC, supports the usefulness of the TEAC for tissue homogenates. The usefulness of this assay is further supported by the observation of a consistent increase in TEAC levels, along with the anticipated increase in glutathione levels (Huang et al., 2000) in brain tissue from ApoE-deficient mice versus normal mice.

While the values obtained by the TEAC assay are not designed to provide information on the full complement of antioxidants present, comparative differences observed between brain homogenates of genetically identical groups when the only difference is the presence or absence of a non-antioxidant dietary compound and between genetically different groups receiving identical diets, support that the observed differences in TEAC values reflect a change in tissue antioxidant concentrations. These findings indicate that the TEAC assay, originally utilized for analyses of plasma and other solutions (Miller et al., 1993), is also useful for determination of overall antioxidant levels in tissue homogenates. While the studies herein have been confined to brain tissue, there is no apparent reason why this assay could not be utilized for other tissues. A similar assay, the oxygen radical absorbance capacity assay (Wayner et al., 1985), also originally designed for analysis of plasma and other solutions, has also been adapted to analysis of tissue samples (Growdon, 2001). Such assays may therefore be particularly useful for monitoring alterations in endogenous antioxidant levels in therapeutic efforts to reduce oxidative stress and resultant damage in AD.

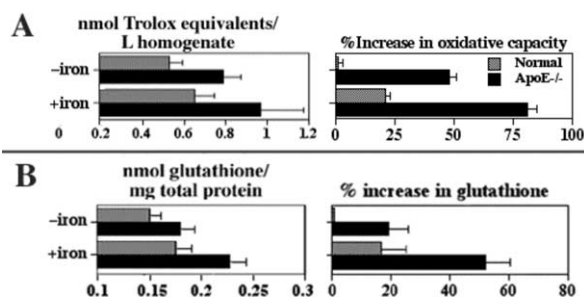


Fig. 1. Total antioxidant and glutathione levels in brain tissue homogenates from normal and ApoE-deficient mice under various dietary regimens. Normal and ApoE<sup>-/-</sup> mice received a basal diet containing or lacking iron for 1 month. Total brain tissue was harvested and analyzed for (panel A) total antioxidant capacity by TEAC or (panel B) total glutathione by HPLC as described in Section 2. Values in panel (A) represent the mean  $\pm$  S.E.M. nmol Trolox equivalents/l homogenate or the mean percentage increase  $\pm$  S.E.M. in Trolox equivalency units for mice receiving iron versus those not receiving iron. Values in panel (B) represent the mean  $\pm$  S.E.M. nmol glutathione/mg total protein or the mean percentage increase versus  $\pm$  S.E.M. in glutathione for mice receiving iron versus those not receiving iron. Data are compiled from greater than or equal to two independent experiments, with  $n = 3-4$  mice per diet per experiment.

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