Strain-Specific Differences in the Expression and Activity of Ogg1 in the CNS

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The expression and activity of 8-oxoguanosine DNA-glycosylase (Ogg1), a key enzyme responsible for the clearance of the oxidized DNA base 8-hydroxy-2'-deoxyguanosine (oxo⁸dG), was determined in the cerebellum (CB) and the caudate and the putamen (CP) of male Balb/c, ICR, and C57BL/J mice. There was no significant difference in the protein expression of Ogg1 in the CB or CP. The activity of Ogg1 was not significantly different in the CB; however, in the CP of ICR mice, the activity of Ogg1 was 34% and 31% lower than Balb/c and C57BL/J, respectively. In contrast, the levels of oxo⁸dG in the CB and CP of C57BL/J mice were nearly twice as high as the values in both regions of Balb/c and ICR mice. The activity of superoxide dismutases (SOD) appeared to account for the differences in the levels of oxo⁸dG in the C57BL/J strain. Total SOD in the C57BL/J strain was two- and fourfold higher in the CB and CP, respectively, versus the other strains. These results suggest that the enhanced vulnerability of the C57BL/J strain to neurotoxicants may not be due to a decreased capacity for DNA repair, but rather, the significantly higher activity of SODs, which may cause these pathways to become more readily saturated.

DNA damage DNA repair Neurodegenerative disease Striatum

HUMAN populations typically display a range of inherent sensitivities to radiation and chemical-induced cellular changes. Given a common chemical insult, some individuals develop associated health problems, whereas others remain clinically free from all related effects of exposure (17). Even within specific populations of exposed individuals such as pesticide applicators, the age of onset and the extent and severity of neurological problems often vary among individuals (2). Such variability in host response may be due, in part, to inherent differences between individuals to monitor and repair damaged sites induced in their genetic material by exogenous and endogenous agents.

This type of variability in response, commonly encountered in the laboratory setting, as well as the choice of animal or strain of animal, can have a dra-

matic impact on the observed outcome for chemical exposures (14,15). For example, when rats are administered doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) comparable to those used in mice, rats do not exhibit any significant dopaminergic degeneration (11,12). Moreover, the optimal neurotoxic effects of MPTP are seen in the C57BL/J strain of mouse (20). When Balb/c and C57BL/J mice are treated with MPTP, neostriatal dopamine depletion is greatest in the C57BL/J strain (–85%) versus the Balb/c strain (–58%). Furthermore, a loss of tyrosine hydroxylase immunoreactivity is only observed in the substantia nigra of C57BL/J animals (–25%) (21). The apparent susceptibility of this strain was initially attributed to a greater density of monoamine oxidase type-B in the basal ganglia and substantia

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nigra, rather than a differential distribution of the enzyme, compared to other strains (25). This may account for an increased capacity for bioactivating MPTP to its neurotoxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺); however, it fails to explain why C57BL/J mice are more susceptible to MPTP toxicity, considering the tissue concentration of MPP⁺ does not appear to be the determining factor for vulnerability of dopaminergic and noradrenergic neurons to MPTP (24). High concentrations of MPP⁺ have been detected in the striatum of brown Norway rats without degenerative effects on dopaminergic neurons (26). Additional studies have shown that there is not a significant difference in the capacity of neostriatal synaptosomes to sequester MPP⁺ between Ace Swiss-Webster, CD-1, or C57BL/J mice (10).

A possible explanation for differences observed between animal species or strains of mice to MPTP toxicity may be explained by regional/cellular differences in the capacity to repair DNA. The objective of the present study was to characterize the protein expression, activity, and substrate levels (oxo⁸dG) for Ogg1 in the cerebellum and the caudate putamen of three commonly used mouse strains: Balb/c, ICR, and C57BL/J.

MATERIALS AND METHODS

Preparation of Samples

All reagents utilized in this study were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise indicated. Male Balb/c, ICR, and C57BL/J mice, weighing around 25 g, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in an air-conditioned animal room, with a 12-h light/dark schedule, and allowed free access to diet and drinking water. Animals were acclimated for 7 days and then killed by decapitation. Brains were rapidly removed and placed in ice-cold saline. Using a dissecting microscope, the cerebellar peduncles were cut with a pair of fine-pointed curved forceps. The cerebral hemispheres were separated with a sagittal cut along the ponto-medullary junction. The caudate and putamen were removed after laying open the hemispheres. The samples were snap frozen in liquid nitrogen and stored at -70°C until time of assay. All of the following assays were performed in parallel to ensure that the values obtained were due to regional or strain-specific differences.

Oxo⁸dG Repair Assay

The extraction of DNA glycosylases from brain tissues was performed as described previously (3).

Briefly, brain tissue was homogenized at 4°C in buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM spermine, 0.5 mM spermidine, 50% glycerol, and 1.3% protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Homogenates were rocked for 30 min after addition of 1/10 volume 2.5 M KCl and spun at 14,000 rpm for 30 min. The supernatant was aliquoted and stored at -70°C until time of assay. The pellet was saved and used for isolation of genomic DNA and antioxidant enzymes. Protein concentration was measured using the bicinchoninic acid (BCA) method (22).

Twenty picomoles of synthetic probe containing oxo⁸dG (Trevigen, Gaithersburg, MD) was labeled with ³²P (adenosine 5'-triphosphate [γ -³²P]) (ICN Pharmaceuticals, Inc., Costa Mesa, CA) at the 5' end using polynucleotide T4 kinase (Boehringer Mannheim, Germany). For the nicking reaction, protein extract (30 μg) was mixed with 20 μl of a reaction mixture containing 0.5 M HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]), 0.1 M EDTA, 5 mM DTT, 400 mM potassium chloride (KCl), purified bovine serum albumin (BSA, New England Biolabs, Inc., Beverly, MA), and labeled probe (approx. 2000 cpm). The reaction was carried out at 37°C for 2 h and stopped by placing the samples in ice. A 20- μl aliquot of loading buffer containing 90% formamide, 10 mM NaOH, and Blue-Orange dye (Promega, Madison, WI) was added to each sample. After 5 min of heating at 95°C , the samples were chilled and loaded into a polyacrylamide gel (20%) with 7 M urea and 1 $\times$ Tris-Borate-EDTA (TBE), and run at 400 mV for 2 h. Gels were quantified using Biorad 363 phosphorimager system and analysis software. Activity to repair oxo⁸dG was determined and expressed as percent of substrate cleaved.

DNA Isolation

Procedures for extraction and purification of DNA and measurement of oxo⁸dG levels were based on minor modifications of published methods (3). Briefly, the pellet obtained from the DNA glycosylase extraction was placed in 10 mM ethylenediamine tetraacetic acid (EDTA), vortexed, and centrifuged at 3500 rpm for 5 min. The supernatant was saved for the measurement of superoxide dismutase activity. The pellets were then placed in a water bath at 37°C for 15 min and subsequently placed at -70°C for 15 min. This was repeated two additional times, and then the pellets were treated with DNAase-free RNAase followed by digestion with proteinase K. The protein fraction was separated from DNA by three consecutive organic extractions. The DNA was precipitated by adding two volumes of ethanol (with respect to

the aqueous volume) and incubated overnight at -20°C . The purity of the DNA was determined by the absorbance of an aliquot of the sample at 260 vs. 280 nm.

Measurement of Oxo⁸dG

Analysis of the ratio of oxo⁸dG/2-deoxyguanosine (2-dG) was performed as described previously (5). Purified DNA was prepared for HPLC analysis by resolving it into deoxynucleoside components. The DNA was digested with nuclease P1 and treated further with alkaline phosphatase. The deoxynucleoside preparation was then ready for HPLC analysis. The amounts of oxo⁸dG and 2-dG were calculated by comparing the peak area of oxo⁸dG and 2-dG obtained from the enzymatic hydrosylate of the DNA sample to a calibration curve for both compounds. Levels of oxo⁸dG in the samples were expressed relative to the content of 2-dG [e.g., the molar ratio of oxo⁸dG/2-dG (fmol oxo⁸dG/nmol of 2-dG)]. Because 1 μg of DNA contains 0.648 nmol of 2-dG, 1 fmol/nmol 2-dG is equivalent to 1.54 fmol/ μg DNA. For the HPLC system, the mobile phase was 100 mM sodium acetate, pH 5.2, with 5% methanol. Oxo⁸dG was detected by an electrochemical detector (ESA Coulochem Model 5100A) using a glassy carbon-working electrode at an applied potential of +0.4 V. 2-dG was detected in the same sample by absorbance at 260 nm using a Perkin Elmer 785A Programmable Absorbance Detector (Perkin Elmer, Norwalk, CT) arranged in series with the electrochemical detector. Data were recorded, stored, and analyzed on a PC Pentium computer using ESA 500 Chromatography Data System Software.

Measurement of SOD

SOD activity was measured according to a previously described procedure based on the SOD-mediated inhibition of nitrite formation from hydroxylammonium in the presence of superoxide anion generators (4,8). Aliquots of the supernatants obtained from the DNA isolation step were used for the determination of SOD activity by incubating the homogenate with xanthine and hydroxylamine chloride. The reaction was initiated by the addition of xanthine oxidase. An aliquot of the incubation mixture was added to a mixture of sulfanilic acid and α -naphthylamine, and the absorbance was read at 529 nm (Ultrospec III spectrophotometer; Pharmacia LKB Biochrom, Cambridge, England). Potassium cyanide (5 mM) was added to parallel tissue homogenates and incubated for 20 min at room temperature to allow for the determination of Mn-SOD activity. The differences between the total and potassium cyanide in-

hibited enzyme activities were used to discriminate between Cu/Zn- and Mn-SOD activities. The activity of SOD in the samples was determined from a calibration curve of the percentage of inhibition of nitrite formation versus SOD activity, which was constructed using known amounts of purified SOD containing 3500 U/mg of protein. One unit of enzyme activity was defined as the amount of SOD required to reduce cytochrome *c* by 50% in the coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume. The amount of protein in the samples was determined using the BCA protein kit. Data are expressed as units of SOD activity per milligram of protein.

Protein Expression and Detection

Protein expression was performed as previously reported (23). Protein (20 μg) of each sample was boiled for 5 min in loading buffer [40 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β -mercaptoethanol, 4% sodium dodecyl sulfate (SDS), and 0.2% bromophenol blue]. The proteins were resolved on 10–12% bis/acrylamide gel by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes using a Biorad Semi-Dry Transblot apparatus. The membranes were blocked overnight at 4°C with 5% dry milk, Tris-buffered saline (TBS) polyoxyethylenesorbitan monolaurate (Tween 20) solution (200 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.04% Tween 20). After washing 1×15 min with TBS-Tween, the membranes were incubated with a 1:1000 dilution of anti-mOgg1 (Alpha Diagnostic International, Inc., San Antonio, TX) in 1% dry milk, TBS-Tween overnight at 4°C . After washing 3×10 min with TBS-Tween, the membranes were incubated with a 1:2000 dilution of goat anti-rabbit HRP-conjugated antibody (Amersham Biosciences Corp., Piscataway, NJ) in 1% dry milk, TBS-Tween for 1 h at room temperature. After washing 3×15 min with TBS-Tween, the blots were developed by ECL (Amersham Biosciences Corp.). The amounts of the respective proteins were quantified by densitometric analysis of radiographic films using Gel-base software (UVP video-image recorder/analysis).

Data Analysis and Statistics

One-way ANOVA was used to compare differences in Ogg1 activity, oxo⁸dG, and Cu/Zn- and Mn-SOD activities in brain regions, followed by Student-Newman-Keuls posttest. Statistical tests were performed with Graphpad statistical software (Sorrento, CA). A probability value less than 0.05 was considered to be significant.

RESULTS

Regional analysis of Ogg1 activity in the cerebellum of male ICR, Balb/c, and C57BL/J mice did not reveal any significant differences (Fig. 1). However, ICR mice had a 66% lower level of Ogg1 activity in the caudate putamen (Fig. 1). The protein levels of Ogg1 were not significantly different by mouse strain or by brain region (Fig. 2).

To determine the functional relevance of these differences, levels of the primary substrate for Ogg1 (oxo⁸dG) were determined by HPLC-EC. There were no region or strain-specific differences in oxo⁸dG levels between the ICR and Balb/c strains; however, the C57BL/J strain had significantly higher levels of oxo⁸dG in the cerebellum (1.98- and 2.2-fold vs. Balb/c and ICR, respectively) and caudate putamen (1.82- and 2.7-fold vs. Balb/c and ICR, respectively) (Fig. 3).

SODs generate hydrogen peroxide, which can trans-

Figure 1. Ogg1 activity in the cerebellum and caudate putamen of Balb/c, ICR, and C57BL/J mice. No significant differences were observed between strains in the cerebellum. The activity of Ogg1 in the caudate putamen of ICR mice was significantly lower than Balb/c and C57BL/J. Data are expressed as the mean $\pm$ SEM, $n = 4$. *Statistically significant difference, $p < 0.05$.

Figure 2. Western blot analysis of Ogg1 in the cerebellum and caudate putamen of Balb/c, ICR, and C57BL/J mice. No significant differences were observed between cerebellum or the caudate putamen. Data are expressed as the mean $\pm$ SEM, $n = 4$.

verse cellular membranes and cause increases in both mitochondrial and nuclear oxo⁸dG levels. Because of this, the activities of the cytosolic Cu/Zn-SOD and the mitochondrial Mn-SOD were determined. Total SOD in the C57BL/J strain was two- and fourfold higher in the cerebellum and caudate putamen, respectively, versus the other strains (data not shown). Cu/Zn-SOD was significantly elevated by greater than 2.45-fold in both the cerebellum and caudate pu-

Figure 3. oxo⁸dG levels in the cerebellum and caudate putamen of Balb/c, ICR, and C57BL/J mice. The levels of oxo⁸dG in both the cerebellum and caudate putamen were significantly higher in the C57BL/J strain than Balb/c or ICR. Data are expressed as the mean $\pm$ SEM, $n = 4$. *Statistically significant difference, $p < 0.05$.

tamen of C57BL/J mice versus Balb/c and ICR mice (Fig. 4). In contrast, there was no significant difference in Mn-SOD activity between strains in the cerebellum; however, the C57BL/J strain had a greater than 6.65-fold increase in Mn-SOD activity in the caudate putamen compared with Balb/c and ICR mice (Fig. 5).

DISCUSSION

A possible explanation for differences observed between animal species or strains to MPTP toxicity may be explained by regional/cellular differences in antioxidant or DNA repair enzymes, as recent evidence has shown that the levels of oxo⁸dG in different brain regions of C57BL/J mice are significantly correlated with the activity of Ogg1 (3). Although this relationship was observed in tissue homogenates containing both actively dividing (glia) and differentiated cells (neurons), it has since been determined

that an inverse relationship between Ogg1 activity and oxo⁸dG exists in terminally differentiated PC12 cells challenged with the organochlorine pesticide, dieldrin. However, this relationship was not observed in actively dividing cells (23). This difference in response may be attributed to higher basal levels of Ogg1 activity in actively dividing cells, because there is a decrease in both Ogg1 protein expression and activity, and a corresponding increase in oxo⁸dG content and Mn-SOD activity, when PC12 cells are differentiated with nerve growth factor (J. Sanchez-Ramos; unpublished findings).

Subsequent studies with C57BL/J mice have indicated that Ogg1 activity is inducible in specific brain regions with low basal levels of activity (cerebellum, cortex, and pons/medulla) and remains unchanged in those regions with high basal levels of activity (hippocampus, caudate/putamen, and midbrain) (6). The present comparison of three different mouse strains (ICR, Balb/c, and C57BL/J) of a region with low Ogg1 activity (cerebellum) versus a region with high Ogg1 activity (caudate/putamen) revealed that C57BL/J mice have nearly twice the basal levels of oxo⁸dG in both

Figure 4. Cu/Zn-SOD activity in the cerebellum and caudate putamen of Balb/c, ICR, and C57BL/J mice. Data are expressed as the mean $\pm$ SEM, $n = 4$. *Statistically significant difference, $p < 0.05$.

Figure 5. Mn-SOD activity in the cerebellum and caudate putamen of Balb/c, ICR, and C57BL/J mice. Data are expressed as the mean $\pm$ SEM, $n = 4$. *Statistically significant difference, $p < 0.05$.

regions compared with ICR and Balb/c mice. This difference was not reflective of a decreased level of Ogg1 activity or protein expression in C57BL/J mice versus ICR or Balb/c. However, the activity of Cu/Zn-SOD was significantly higher in the cerebellum and caudate/putamen of C57BL/J mice and Mn-SOD activity was significantly higher (greater than sixfold) in the caudate/putamen of the C57BL/J strain.

Based on these results, it is suggested that the enhanced susceptibility of the C57BL/J strain to neurotoxicants (e.g., MPTP) may be due to saturation of BER pathways. In mitochondria, superoxide anion is the primary product of unreduced oxygen, which re-

sults in the production of hydrogen peroxide either enzymatically by Mn-SOD or by spontaneous disproportionation (9). Inhibition of complex I by MPP⁺ has been shown to result in an increased production of superoxide anion (16). Therefore, the enhanced susceptibility of C57BL/J mice to MPTP may be the result of the high basal activity of Mn-SOD, as it would serve to increase the production of hydrogen peroxide and, ultimately, levels of oxidative DNA damage.

The increased activity of Cu/Zn-SOD in C57BL/J mice further supports this notion, as enhanced Cu/Zn-SOD activity has been linked with Down's syndrome, a disease characterized by premature aging and neurological impairment (1). In neural tissues, Cu/Zn-SOD and glutathione peroxidase are the predominant oxygen scavenging enzymes (13,18). A linear increase in Cu/Zn-SOD activity has been reported with aging in mouse brains; however, there was no detectable increase in glutathione peroxidase with aging, as is the case in neural tissues of Down's syndrome patients (7).

Because the striatum is predisposed to oxidative stress from dopamine metabolism and autooxidation, the enhanced activity of SODs in this region of the C57BL/J strain may preclude its susceptibility to neurotoxicants. It is interesting to note, however, that the ICR strain of mouse has previously been shown to have a higher brain dopamine concentration versus the Balb/c and C57BL/J strain (19), indicating that the enhanced activities of SODs in the C57BL/J mice may not be reflective of a compensatory response to increased levels of reactive oxygen species generated from the metabolism and autooxidation of dopamine.

The current findings demonstrate that the higher basal level of oxidative stress observed in the C57BL/J strain may account for their enhanced sensitivity to neurotoxicants. Our data indicate that because of the differences that exist between the strain of animal and the capacity for DNA repair and antioxidant enzymes, special care should be taken when interpreting and reporting neurotoxicological findings.

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