Oxidative Stress Following Traumatic Brain Injury in Rats: Quantitation of Biomarkers and Detection of Free Radical Intermediates

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Abstract: Oxidative stress may contribute to many pathophysiologic changes that occur after traumatic brain injury. In the current study, contemporary methods of detecting oxidative stress were used in a rodent model of traumatic brain injury. The level of the stable product derived from peroxidation of arachidonyl residues in phospholipids, 8-epi-prostaglandin F_2α, was increased at 6 and 24 h after traumatic brain injury. Furthermore, relative amounts of fluorescent end products of lipid peroxidation in brain extracts were increased at 6 and 24 h after trauma compared with sham-operated controls. The total antioxidant reserves of brain homogenates and water-soluble antioxidant reserves as well as tissue concentrations of ascorbate, GSH, and protein sulfhydrides were reduced after traumatic brain injury. A selective inhibitor of cyclooxygenase-2, SC 58125, prevented depletion of ascorbate and thiols, the two major water-soluble antioxidants in traumatized brain. Electron paramagnetic resonance (EPR) spectroscopy of rat cortex homogenates failed to detect any radical adducts with a spin trap, 5,5-dimethyl-1-pyrroline N-oxide, but did detect ascorbate radical signals. The ascorbate radical EPR signals increased in brain homogenates derived from traumatized brain samples compared with sham-operated controls. These results along with detailed model experiments in vitro indicate that ascorbate is an important antioxidant in brain and that the EPR assay of ascorbate radicals may be used to monitor production of free radicals in brain tissue after traumatic brain injury. Key Words: Traumatic brain injury—Oxidative stress—Ascorbate—Spin traps—Thiols—8-epi-Prostaglandin F_2α. J. Neurochem. 75, 2178–2189 (2000).

Interplay of three major deleterious pathways—glutamate excitotoxicity, Ca^{2+} overload, and oxidative stress—is believed to be responsible for the damage and neuronal death following traumatic brain injury (TBI) (Juurlink and Paterson, 1998). It has been suggested that oxidative stress plays a key role both in primary damage following acute TBI (Kontos and Povlishock, 1986; Ikeda and Long, 1990) and in secondary deleterious processes associated with inflammatory mediators and neutrophil-mediated inflammation (Feuerstein et al., 1997; Juurlink and Paterson, 1998). Furthermore, reactive oxygen species may accelerate axonal damage after TBI (Povlishock and Kontos, 1992). Although this hypothesis has been strongly supported by successful use of different classes of antioxidants and spin traps as protectors against TBI (Chan et al., 1987; Ikeda and Long, 1990; Sen et al., 1994; Inci et al., 1998; Lewen and Hillered, 1998; Zhang et al., 1998), the sources and mechanisms of oxidative stress induced by TBI, however, have not been yet identified (Awashtii et al., 1997). Furthermore, despite the prevailing opinion on the important role of free radicals in TBI, the direct experimental data supporting this notion remain controversial.

Cyclooxygenase is the enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. The enzyme also has peroxidase activity and can induce formation of radicals and dopamine quinones independent of its...
metabolism of arachidonic acid (Kukreja et al., 1986; Hastings, 1995). Cyclooxygenase-2 (COX-2) is the inducible isoenzyme of the enzyme that has high levels of expression in brain. COX-2 transcription is induced by synaptic activity (Yamagata et al., 1993). Excitatory amino acids are released into the extracellular space after TBI and thus may produce secondary injury by excitotoxicity. The neuronal excitation elicited by these excitatory amino acids would be expected to induce expression of COX-2. Indeed, increased COX-2 expression has been observed in rodent brain trauma models (Dash et al., 2000). COX-2 activity has been shown to exacerbate injury in brain ischemia and spinal cord injury (Nakayama et al., 1998; Nogawa et al., 1998; Resnick et al., 1998). Because COX-2 activity may be associated with the production of radicals, we hypothesized that COX-2 activity could contribute to oxidative stress after TBI.

Detection of short-lived radicals in biological systems is feasible through use of spin traps, molecules reacting with highly reactive radicals to produce relatively stable radical adducts readily detectable by their characteristic features in electron paramagnetic resonance (EPR) spectra (Buettner and Mason, 1990). Numerous EPR spin trap studies have reported qualitative indications of some kind of enhanced free radical generation at different intervals after or during TBI, but no attempts have been made to characterize quantitatively the amounts and time course of free radical production and correlate them with neuronal damage (Awasthi et al., 1997; Nishio et al., 1992). Moreover, nitrooxide spin adducts that are formed on interaction with the most commonly used spin traps, such as 5,5-dimethyl-1-pyrroline N-oxide (DMPO), α-phenyl-N-tert-butyl nitroxide, and α-(4-pyridyl-1-oxide)-N-tert-butyl nitroxide, can be reduced by endogenous redox agents to yield EPR-silent hydroxylamines, thereby confounding any quantitative determinations. In particular, ascorbate was found to reduce rapidly nitrooxide radical adducts to hydroxylamines (Sentjurc and Mason, 1992; Stoyanovsky and Cederbaum, 1998). In the course of this reaction, ascorbate undergoes one-electron oxidation to form ascorbate radical. It is not surprising that augmented EPR signals of ascorbate radicals have been detected in EPR spectra of different tissues experiencing oxidative stress, including traumatized brain tissue (Awasthi et al., 1997) as well as microdialysis samples (Kihara et al., 1995), rather than signals from spin adducts of oxygen radicals. Given that ascorbate concentrations may be as high as 0.9–2.0 mM in the brain (and in some regions they may even be as high as 10 mM) (Grunewald, 1993; Rice and Russo-Menna, 1998), the interactions of spin adducts with ascorbate may be particularly important for assessment of TBI-induced free radical generation in the brain. To the best of our knowledge, systematic studies of the effects of endogenous ascorbate on free radical production in traumatized brain have not been conducted, although several laboratories have reported elevated levels of ascorbate radicals in brain tissue following TBI (Kihara et al., 1995; Awasthi et al., 1997).

In the present study, we used an established controlled cortical impact model of TBI (Kochanek et al., 1995) to assess several important biomarkers of oxidative stress. We characterized endogenous oxidative stress in lipids by using the most reliable marker for in vivo lipid peroxidation (LPO), derivatives of F2-isoprostane (8-epi-PGF2α) (Morrow et al., 1992), and complemented this by measurements of levels of fluorescent end products of LPO (Dillard and Tappel, 1984). For proteins, we monitored oxidation of SH groups using a very sensitive fluorogenic maleimide reagent, ThioGlo-1 (Shvedova et al., 2000). We also performed assays of total antioxidant reserves as well as of major individual water- and lipid-soluble antioxidants (ascorbate, GSH, and vitamin E) to characterize fully TBI-induced oxidative damage.

Based on our findings of enhanced oxidative stress in TBI, we further attempted to reveal TBI-induced free radical production using spin trap EPR measurements. We chose a spin trap, DMPO, that detects spin adducts of oxygen radicals as well as S-centered radicals [thyl radicals (GS radicals)] of GSH (Buettner and Mason, 1990). Despite extensive attempts we were unable to detect any endogenous generation of DMPO adducts with either oxygen radicals or GS radicals. Instead, we found that ascorbate radical production was enhanced in brain after TBI. We then conducted detailed studies in vitro to establish the conditions in brain homogenates that are necessary for reliable detection of DMPO spin adducts with hydroxyl radicals (•OH radicals) and GS radicals. We present evidence that high levels of endogenous ascorbate and its interactions with oxygen radicals preclude any possible EPR or HPLC detection of DMPO adducts in brain tissue. There is a depletion of brain ascorbate stores after TBI that is prevented by pretreatment with a selective COX-2 inhibitor, SC 58125. Thus, the readily EPR-detectable formation of ascorbate radicals and depletion of antioxidants may be used to study the production and source of oxidative stress in TBI.

MATERIALS AND METHODS

Materials

8-epi-PGF2α was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). 1-Pyrenyl diazomethane (PDAM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). The maleimide-based thiol reagent ThioGlo-1 was obtained from Covalent Associates Inc. (Woburn, MA, U.S.A.). 2,2′-Azobis(2-aminodinopropane) dihydrochloride (AAPH) was supplied by Waco Chemicals (Richmond, VA, U.S.A.). Luminol, etoposide (VP-16), tyrosinase, horseradish peroxidase, ascorbate oxidase, DMPO, deferoxamine mesylate, diethylenetriaminepentaacetic acid (DTPA), sodium dodecyl sulfate (SDS), phenol, H2O2, acetanilide, ethyl acetate, hexane, tetrahydrofuran, dimethyl sulfoxide, chloroform, methanol, Tris(hydroxymethyl)aminomethane (Sigma 7-9), ferrous sulfate, GSH, NaCl, NaH2PO4, and NaH2PO4 were purchased from Sigma (St. Louis, MO, U.S.A.). Acetic acid and ascorbate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Chelex 100 ion-exchange resin was obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).
Head trauma model

The experimental protocol was approved by the appropriate Institutional Animal Care and Usage Committee of the University of Pittsburgh. The controlled cortical impact model was used as previously described (Kochanek et al., 1995). In brief, male Sprague–Dawley rats (weighing 300–350 g; Charles River Laboratories) were intubated and ventilated with 1.1% isoflurane/66% nitrous oxide, with the balance being O2. A craniotomy was made over the left parietal cortex. A temperature probe was inserted through a small burr hole into the right parietal lobe for brain temperature monitoring. Injury was induced using a CCI device (Dixon et al., 1991) with the following parameters: depth, 5 mm; velocity, 4.0 ± 0.2 m/s; and duration of deformation, 50 ms. The bone flap was replaced and sealed with dental cement, and the scalp incision was closed. All animals were killed by an overdose of pentobarbital followed by decapitation with a guillotine, which is consistent with the recommendations of the American Veterinary Medicine Association. The rats were decapitated 6, 24, or 72 h after TBI. Controls consisted of sham-operated rats that underwent identical anesthesia and surgery except that controlled cortical impact was not performed. Ipsilateral and contralateral cortex and hippocampus were removed and snap-frozen in liquid nitrogen.

To study the effect of a COX-2 inhibitor, SC 58125, on antioxidant reserves in brain cortex and hippocampus, rats were given the inhibitor at a dose of 30 mg/kg body weight, p.o., 1 h before TBI. The rats were decapitated 24 h after TBI. To detect TBI-induced oxygen radicals as well as S-centered (thyl) radicals, rats were treated with a spin trap, DMPO. DMPO dissolved in 1 ml of saline was injected intravenously into rats at a dose of 25 mmol/kg of body weight 5 min before TBI. The rats were decapitated 1.5, 4, or 6 h after TBI.

HPLC assay of 8-epi-PGF$_2\alpha$

Brain homogenates were mixed with acetonitrile (1:1, vol/vol) and centrifuged at 10,000 g for 5 min. Lipids were extracted from pellet using the Folch procedure and hydrolyzed at alkaline conditions (Morrow et al., 1992). 8-epi-PGF$_2\alpha$ was isolated from a hydrolysate by solid-phase extraction and HPLC. In brief, lipid hydrolysate was diluted in 0.1 M KH$_2$PO$_4$ to 5 ml and passed through a 1 ml Sep-Pak ODS cartridge (Waters Associates, Milford, MA, U.S.A.) previously washed with methanol and water. The cartridge was then washed successively with 2 ml of water and 2 ml of acetonitrile/water/acetic acid (28:71:9.0:1, by volume) and dried by suction. Crude 8-epi-PGF$_2\alpha$ was obtained from the cartridge by elution with 2 ml of 80% methanol. The eluate was evaporated under N$_2$ and dissolved in 80% methanol, and 8-epi-PGF$_2\alpha$ was separated from free fatty acids by HPLC using a Hypersil ODS column (200 × 4.6 mm; particle size, 5 μm). A Shimadzu (Kyoto, Japan) HPLC system was used with an LC-600 pump and SPD-10AV UV detector (absorbance at 205 nm). The eluent was acetonitrile/water/acetic acid (28:71:9.0:1, by volume) at a flow rate of 1 ml/min. Under these conditions the retention time for 8-epi-PGF$_2\alpha$ was 17–21 min. 8-epi-PGF$_2\alpha$ was used as a standard. The collected fraction of 8-epi-PGF$_2\alpha$ was diluted four times with water and subjected to solid-phase extraction one more time as described above. 8-epi-PGF$_2\alpha$ was derivatized using PDAM (Nimura et al., 1988), and the 1-pyre-nylmethyl ester of 8-epi-PGF$_2\alpha$ was detected. In brief, the fraction containing 8-epi-PGF$_2\alpha$ was dissolved in methanol and applied on a 4 × 4 mm paper square (Whatman, Maidstone, U.K.) under N$_2$ and treated with 50 μl of PDAM (0.5 mg/ml in hexane/ethyl acetate, 1:1, vol/vol) for 12 h at 35°C in anaerobic conditions. To decompose any excess of PDAM, at the end of incubation the solution was changed to 100 μl of hexane/acetic acid (99:1, vol/vol), and samples were kept at 35°C for 30 min. The paper square was washed three times with 250 μl of hexane/ethyl acetate (9:1, vol/vol). A 1-pyre-nylmethyl ester of 8-epi-PGF$_2\alpha$ was extracted by ethyl acetate/acetic acid (97:3, vol/vol) and assayed by HPLC using a Luna phenylhexyl column (250 × 4.6 mm; particle size, 5 μm; Phenomenex, Torrance, CA, U.S.A.). A Shimadzu HPLC system was used with an LC-600 pump and RF-551 fluorescence detector (excitation at 324 nm and emission at 395 nm). The eluent was methanol/tetrahydrofuran/water (43:23:34, by volume) at a flow rate of 1 ml/min. Under these conditions the retention time for 8-epi-PGF$_2\alpha$ was 24 min. Data acquired were exported from detectors using Shimadzu EZChrom software.

Assay of fluorescent end products of LPO

Lipids were extracted from brain homogenates using the Folch procedure, and lipid phosphorus was quantified as previously described (Shvedova et al., 2000). Levels of fluorescent end products of LPO were estimated by fluorescence emission intensity at 440 nm as described (Dillard and Tappel, 1984).

Fluorescence assay of protein SH groups and GSH

The concentration of total SH groups (GSH plus protein sulfhydryl groups) in brain homogenates was determined using Thioglo-1, a maleimide reagent, which produces a highly fluorescent product on its reaction with SH groups. To homogenates of brain tissue containing 15–30 μg of protein/ml, Thioglo-1 was added to a final concentration of 10 μM (in dimethyl sulfoxide solution). GSH content was estimated by an immediate fluorescence response observed on addition of Thioglo-1 to the brain homogenate (Shvedova et al., 2000). A standard curve was established by addition of GSH (0.04–4.0 μM) to 50 mM disodium phosphate buffer (pH 7.4) containing 10 μM Thioglo-1. Levels of total protein sulfhydrys were determined as an additional fluorescence response after addition of SDS (4 mM) to the same homogenate. In separate experiments, we tested the effects of SDS on the fluorescence response of Thioglo-1 and found that within the error of our measurements the fluorescence intensity from the GSH–ThioGlo-1 adduct was not changed on addition of SDS to a final concentration of 4.0 mM (when the background response of Thioglo-1 in the absence of GSH was recorded in the presence of SDS). A Shimadzu RF-5301PC spectrophotofluorometer was used in the assay of fluorescence at 388 nm (excitation) and 500 nm (emission). The data acquired were exported from the spectrophotofluorometer using Shimadzu RF-5301PC Personal Fluorescence Software.

Chemiluminescence measurements of total antioxidant reserve

Total antioxidant reserve in brain homogenates was assayed by chemiluminescence produced in the presence of luminol and a peroxyl radical generator as described (Tyurina et al., 1995). A water-soluble azo-initiator, AAPH, was used to produce peroxyl radicals at a constant rate (Niki, 1990) and oxidized luminol (400 μM) in 50 mM disodium phosphate buffer (pH 7.4) at 37°C. The reaction was started by addition of AAPH (50 mM). A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derivived peroxyl radicals, is observed on addition of brain homogenate (0.1 mg of protein/ml). Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants can be deter-
mined. A Luminescent Analyzer model 633 (Coral Biomedical, San Diego, CA, U.S.A.) was used for determinations.

**EPR measurements**

Water-soluble antioxidant reserves. To estimate water-soluble antioxidant reserves (combined radical scavenging activity caused mainly by ascorbate and thiols), EPR spectral monitoring of tyrosinase-induced oxidation of the hindered phenol VP-16 to its phenoxyl radical was used (Gantchev et al., 1994). For measurements of VP-16 phenoxyl radical formation, VP-16 (500 μM) and tyrosinase (2.8 U) were incubated in 50 mM disodium phosphate buffer containing NaCl (100 mM) and deferoxamine mesylate (100 μM; pH 7.4 at 25°C) in the presence or in the absence of brain homogenates. The phosphate buffer was pretreated with Chelex 100 ion-exchange resin to remove possible transition metal ion contaminants.

Brain homogenates (0.2 mg of protein) were added to the incubation mixture containing VP-16, tyrosinase, and deferoxamine mesylate (100 μM; pH 7.4 at 25°C) in the presence or the absence of brain homogenates. The phosphate buffer was pretreated with Chelex 100 ion-exchange resin to remove possible transition metal ion contaminants. Brain homogenates (0.2 mg of protein) were added to the incubation mixture containing VP-16, tyrosinase, and deferoxamine mesylate (100 μM; pH 7.4 at 25°C) in the presence or the absence of brain homogenates. The phosphate buffer was pretreated with Chelex 100 ion-exchange resin to remove possible transition metal ion contaminants.

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Ascorbate radicals. Sham-operated rats and rats after TBI were decapitated. The brains were quickly removed and homogenized in double-distilled water, and 80 μl of homogenate (2 mg of protein) was immediately used for assay of ascorbate radical formation by EPR using spectrometer parameters as described above (except that field modulation was 0.79 G).

DMPO-OH adduct. We used a spin trap, DMPO, for the study of oxygen-centered free radical formation (superoxide, "OH radicals, and peroxyl radicals) in vivo and in vitro (Buetter and Mason, 1990). DMPO was purified by double recrystallization and stored under nitrogen at ~80°C before use, and the buffer was treated with Chelex 100 ion-exchange resin. EPR spectra were recorded at 25°C from homogenates of rat cortex (0.2 mg of protein) in 80 μl of 50 mM disodium phosphate buffer (pH 7.4) prepared from sham-operated rats and rats after TBI (1.5, 4.0, and 6.0 h). In in vitro experiments the concentration of DMPO ranged from 1 to 200 mM. To remove exogenous ascorbate, homogenates were treated with ascorbate oxidase (2.5 U) for 20 min at 25°C. In experiments where measurements of formation of DMPO-OH adducts were performed in the presence of FeSO₄ (100 μM) and H₂O₂ (1.0 mM), a four-line EPR spectrum with hyperfine splitting constants (αN = 14.8 G and αH = 14.8 G) characteristic of a DMPO-OH adduct was obtained. Spectra of DMPO-OH radical adducts were recorded with the following parameters: center field, 3.35 G; power, 20 mW; field modulation, 0.79 G; sweep width, 50 G; receiver gain, 4,000; time constant, 0.1 s.

**RESULTS**

Biochemical markers of oxidative stress in brain after TBI

8-epi-PGF₂α, 8-epi-PGF₂α derivatives are novel bioactive cyclopentenone PG-like compounds produced in vivo by free radical peroxidation of arachidonyl-containing lipids and represent the most reliable lipid biomarker of oxidative stress (Morrow et al., 1992; Roberts et al., 1998; Chen et al., 1999). The presence of 8-epi-PGF₂α in lipid extracts of rat brain after isolation and derivatization was confirmed by fluorescence HPLC (Fig. 1A). The content of 8-epi-PGF₂α in brain cortex was within the

**Quantification of protein**

The protein concentration in the brain homogenates was determined with the Bio-Rad Protein Assay kit. A standard curve was established by addition of bovine serum albumin to the Bio-Rad kit, and protein content was calculated.

**Statistical evaluation**

Data are mean ± SEM values of at least three experiments. The data were analyzed by ANOVA. A value of p < 0.05 was considered to indicate a significant difference.
protein SH content showed significantly decreased content during recovery from TBI. Analysis of proteolytic factors [67x241]that the levels of different LPO products may change with time after TBI.

Accumulation of fluorescent end products of LPO in rat brain cortex. A: HPLC tracings of 8-epi-PGF\textsubscript{2\alpha} fluorescently derivatized with PDAM (trace a) and lipid extract of the ipsilateral area of rat brain fluorescently derivatized with PDAM (trace b). The arrow shows the 8-epi-PGF\textsubscript{2\alpha} derivative. FAU, fluorescence arbitrary units. B: Content of 8-epi-PGF\textsubscript{2\alpha} in the ipsilateral area of brain obtained from sham-operated rats and TBI-exposed rats (6 and 24 h after TBI). Data are mean ± SEM (bars) values (n = 5). "p < 0.02, **p < 0.05 versus control.

range of 2.6 ± 0.7 pg/μg of phosphorus, in line with previously reported results (Roberts et al., 1998; Pratico et al., 1999). The concentrations of 8-epi-PGF\textsubscript{2\alpha} were increased 10- and 2.5-fold 6 and 24 h after TBI, respectively (Fig. 1B).

Fluorescent LPO products. Accumulation of fluorescent chromophores with a fluorescence emission maximum of ~440 nm is a characteristic feature of terminal stages of free radical oxidation of lipids (Dillard and Tappel, 1984). Measurements of relative fluorescence intensity of total lipid extracts prepared from untraumatized rat brain and from rat brain 6, 24, and 72 h following TBI are presented in Fig. 2A. This shows a progressive accumulation of fluorescent end products of LPO with time after TBI.

Combined, these results indicate that brain lipids represent a target of free radical attack following TBI and that the levels of different LPO products may change during recovery from TBI.

Protein SH oxidation. Free radical attack on proteins results in oxidation of their SH groups. Analysis of protein SH content showed significantly decreased concentrations of protein sulfhydryls in ipsilateral cortex of the rat brain 6, 24, and 72 h after TBI compared with controls. In contralateral cortex, a significant decrease in content of protein sulfhydryls was found only 6 h after TBI (Fig. 2B). Similar but less pronounced changes were detected in hippocampus (Fig. 2C).

Antioxidant status of rat brain after TBI

Total antioxidant reserves. To assess total antioxidant reserves of rat brain, homogenates were subjected to free radical attack from peroxyl radicals generated by AAPH in the presence of a reporter molecule, luminol. This is shown in a control experiment presented in Fig. 3A in which luminol in buffer interacts with peroxyl radicals generated from AAPH to produce light. This response was not observed when either AAPH or luminol was omitted. The chemiluminescence is delayed in the presence of brain cortex homogenate by quenching of peroxyl radicals by endogenous antioxidants, which compete with luminol for reaction with peroxyl radicals (Tyurina et al., 1995). The duration of the lag period produced by brain homogenates from control (sham-operated) rats was significantly greater than that observed in the presence of brain homogenates from rats previously subjected to TBI (Fig. 3A). Calculations, based on the measurements of lag periods and known rates of radical production by AAPH (Niki, 1990), demonstrated that brain cortex (Fig. 3B) and hippocampus (Fig. 3C) homogenates from control rats were able to scavenge ~92–100 nmol of peroxyl radicals/mg of protein. The luminol-enhanced chemiluminescence assay revealed significant decreases (30–40%) in total antioxidant reserves of ipsilateral brain cortex 6 and 24 h after TBI (Fig. 3B), but these had recovered to near normal values after 72 h. In contralateral cortex, a significant depletion of antioxidant reserves was observed only 6 h after TBI (Fig. 3B). Similarly, a significant decrease in antioxidant reserves occurred 6 and 24 h after TBI in ipsilateral hippocampus. This effect of TBI on antioxidant reserves was also observed (although it was less pronounced) in contralateral hippocampus (Fig. 3C).

These data suggest that there is a transient depletion of antioxidant reserves in rat brain immediately following TBI.
TBI and that this is restored during recovery from the injury.

Water-soluble antioxidant reserves. We used EPR spectroscopy to identify radical species produced by reaction of antioxidants with phenoxyl radicals. VP-16 acts as a substrate for oxidation catalyzed by tyrosinase to produce a convenient source of phenoxyl radicals uncontaminated with other radicals capable of oxidizing major water-soluble antioxidants (Kagan et al., 1994; Tyurina et al., 1995). The method permits detection of dynamic interactions of intracellular reductants (mainly ascorbate and thiols) in tissues (Gantchev et al., 1994; Kagan et al., 1994; Tyurina et al., 1995).

Tyrosinase-catalyzed oxidation of VP-16 alone in phosphate buffer yields its phenoxyl radicals with a characteristic EPR spectrum (Fig. 4A, trace b). When ascorbate, VP-16, and tyrosinase were added to the phosphate buffer, the doublet EPR signal of the ascorbate radical was first observed (Fig. 4A, trace a). The ascorbate radical is formed by one-electron oxidation of ascorbate by VP-16/tyrosinase-derived phenoxyl radicals (Kagan et al., 1994). This signal was transient and was replaced by the VP-16 phenoxyl radical signal, which appeared only after the ascorbate radical signal completely disappeared from the spectrum (Fig. 4A, trace b). The time course of changes in signal amplitudes of the two radical species is presented in Fig. 4B. The duration of the ascorbate radical signal was directly related to the concentration of ascorbate added (data not shown). In the absence of VP-16, the amplitude of the ascorbate EPR signal generated in the presence of tyrosinase was not reduced, and the EPR signal of the ascorbate radical persisted for several hours.

When reduced GSH, VP-16, and tyrosinase were added to the phosphate buffer, the VP-16 phenoxyl radical EPR signal could not be detected (Fig. 4C). After a lag period, the duration of which was directly related to the amount of GSH added to the reaction mixture (data not shown), the EPR signal of VP-16 phenoxyl radical appeared and persisted over time. The interval during which no EPR signals were detectable (lag period of 14 min in Fig. 4C) corresponds to thiol-dependent reduction of phenoxyl radicals (Gantchev et al., 1994; Tyurina et al., 1995).

In the presence of brain cortex homogenate (Fig. 4D), the characteristic EPR signal of the VP-16 phenoxyl radical was not observed initially. Instead, the doublet EPR signal of the ascorbate radical was immediately recognized. The persistence of the EPR signal of the ascorbate radical was directly related to the content of endogenous ascorbate in the homogenates and was completely abolished by pretreatment of homogenates with ascorbate oxidase (data not shown). The EPR signal of the ascorbate radical in brain homogenates was also transient and disappeared after ~8–9 min of incubation. No signals were observed in the EPR spectra during the subsequent 7–8 min. After depletion of both endogenous ascorbate and thiols, the VP-16 phenoxyl radical EPR signal was observed, and the amplitude of the spectral line increased progressively over the next 5–7 min as all the antioxidant reserves were depleted. The duration of ascorbate radicals (Fig. 4E) and the respective amounts of available ascorbate (Table 1) as well as lag periods (Fig. 4F) caused by interaction of VP-16 phenoxyl radicals with thiols were significantly diminished 24 h after TBI in both brain cortex and hippocampus.

Individual antioxidants of rat brain after TBI
Analyses of GSH and the major lipid-soluble antioxidant, vitamin E (α-tocopherol), were also performed by fluorescence measurements and HPLC, respectively (Table 1). It can be seen that GSH levels decrease significantly in both regions of brain after 6 and 24 h following TBI. At 72 h the content of these antioxidants is largely restored to control levels. Comparison of the results on GSH loss at 24 h after TBI (Table 1) with those obtained by EPR measurements of VP-16 radicals (Fig. 4F) shows that they are in good quantitative agreement with each other. Similar trends but less pronounced changes were found in samples obtained from contralateral areas of the brain (Table 1).
FIG. 4. Effect of TBI on water-soluble antioxidant reserves in homogenates of ipsilateral rat brain cortex. Water-soluble antioxidant reserves in homogenates of ipsilateral brain cortex obtained from sham-operated (control) rats and TBI-exposed rats were determined by EPR spectral monitoring of tyrosinase-induced oxidation of VP-16 (0.5 mM) to its phenoxyl radical as described in Materials and Methods. A: Typical EPR spectra of ascrobate radical (trace a) and VP-16 phenoxyl radical (trace b) generated by tyrosinase (2.8 U) in 50 mM disodium phosphate buffer, pH 7.4. B: Time course of ascorbate radical concentration (A) and VP-16 phenoxyl radical concentration (B) generated by tyrosinase in 50 mM disodium phosphate buffer, pH 7.4. The ascorbate concentration was 210 μM. C: Time course of VP-16 phenoxyl radical generated by tyrosinase in 50 mM disodium phosphate buffer (pH 7.4) in the presence (●) and in the absence (●) of GSH (330 μM). D: Time course of ascorbate radical (●) and VP-16 phenoxyl radical (●) generated by tyrosinase in the presence of cortex homogenate (3.3 mg of protein/ml). E: Effect of TBI on duration of ascorbate radical generated by tyrosinase and VP-16 in 50 mM disodium phosphate buffer (pH 7.4) in the presence of control (●) and TBI (●) cortex and hippocampus homogenates. Data are mean ± SEM (bars) values (n = 6). †p < 0.05 versus control. F: Effect of TBI on duration of lag period for appearance of VP-16 phenoxyl radical generated by tyrosinase in 50 mM disodium phosphate buffer (pH 7.4) in the presence of control (●) and TBI (●) cortex and hippocampus homogenates. Data are mean ± SEM (bars) values (n = 6). †p < 0.05 versus control.

The tissue content of α-tocopherol remains relatively constant, suggesting that mechanisms for vitamin E recycling in rat brain are very efficient so that there is no net loss of vitamin E at any stage following TBI.

Effects of the COX-2 inhibitor SC 58125 on water-soluble antioxidant reserves in brain cortex and hippocampus of rats after TBI

COX-2 is an inducible enzyme of brain concerned with arachidonic acid oxidation (Kujubu and Herschman, 1992; O’Banion et al., 1992; Feng et al., 1993) and is believed to be an important mediator of cell injury resulting from oxidative stress (Kim et al., 2000). The effect of administration of an inhibitor of COX-2, SC 58125, to rats before TBI on the content of ascorbate and GSH of rat brain 24 h after TBI is shown in Table 2. This shows that the significant reduction in levels of both available ascorbate and GSH in the cortex and hippocampus that results from TBI can be completely prevented by treatment of the animals with this drug. This suggests that COX-2 may be a major source of radicals in brain involved in oxidative stress following TBI.

Detection of free radicals generated in brain after TBI

Because our results clearly indicate that both accumulation of biomarkers of oxidative stress and depletion of antioxidant reserves occur in brain after TBI, we next attempted to directly detect formation of free radicals in the brain after TBI. We expected that oxygen radicals as well as radicals of the major water-soluble antioxidants, GSH and ascorbate, could be detectable as both antioxidants were consumed by TBI. Therefore, we chose a spin trap, DMPO, that forms distinct spin adducts with oxygen radicals as well as with GSH radicals. Monitoring of the ascorbate radical does not require spin traps as its duration is sufficient for direct EPR detection of steady-state concentrations (Buettner and Jurkiewicz, 1993).

We found no discernible DMPO adducts with either oxygen radicals or GSH radicals at any of the intervals studied after TBI. Similarly, no GS-DMPO nitrene was detectable on HPLC chromatograms of extracts from brain samples obtained at 1.5, 4.0, or 6.0 h after TBI (data not shown). Using HPLC we found that DMPO was present in brain tissue at 1.5 h after TBI at concentrations of 840 ± 57 and 785 ± 70 nmol/mg of protein in contralateral and ipsilateral areas of the brain cortex, respectively. Although its concentration decreased at 6.0 h after TBI by 73 and 64% in contralateral and ipsilateral areas, respectively, the concentrations were quite sufficient for detection of DMPO adducts under appropriate conditions. In all cases, however, a pronounced signal of the ascorbate radical was readily detectable in the EPR spectra (Fig. 5A).

We conducted in vitro experiments to identify the conditions compatible with the detection of DMPO adducts in the brain. We found that even in the presence of the potent oxidants Fe2+/H2O2, DMPO-OH adducts in EPR spectra were observed only after pretreatment of the brain cortex homogenates with ascorbate oxidase (Fig. 5B). Similar results were obtained in attempts to identify spin adducts of glutathionyl radicals by DMPO. Both EPR and HPLC assays demonstrated that GS-DMPO adducts were only detectable after elimination of endogenous ascorbate after treatment of samples with ascorbate oxidase (data not shown). Thus, ascorbate dominates the antioxidant response to TBI and precludes detection of hydroxyl and glutathionyl adducts with DMPO.
TABLE 1. Effect of TBI on content of antioxidants in rat brain cortex and hippocampus

<table>
<thead>
<tr>
<th>Antioxidant (nmol/mg of protein)</th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant compound</td>
<td>Control</td>
<td>Ipsilateral</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>63.0 ± 5.6</td>
<td>53.9 ± 3.0</td>
</tr>
<tr>
<td>Glutathione</td>
<td>48.2 ± 3.4a</td>
<td>34.8 ± 4.5a</td>
</tr>
<tr>
<td>Glutathione Control</td>
<td>55.3 ± 4.3</td>
<td>33.3 ± 1.9a</td>
</tr>
<tr>
<td>Glutathione 24 h</td>
<td>65.8 ± 3.1</td>
<td>53.7 ± 3.9</td>
</tr>
<tr>
<td>Glutathione 72 h</td>
<td>6.1 ± 2.0</td>
<td>1.7 ± 0.12</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>2.24 ± 0.21</td>
<td>2.10 ± 0.12</td>
</tr>
<tr>
<td>α-Tocopherol Control</td>
<td>2.05 ± 0.28</td>
<td>1.82 ± 0.16</td>
</tr>
<tr>
<td>α-Tocopherol 24 h</td>
<td>2.00 ± 0.3</td>
<td>1.77 ± 0.21</td>
</tr>
<tr>
<td>α-Tocopherol 72 h</td>
<td>2.08 ± 0.16</td>
<td>2.00 ± 0.16</td>
</tr>
</tbody>
</table>

Data are mean ± SEM values (n = 6).

*a p < 0.05, *p < 0.001 versus control (contralateral cortex or hippocampus from control rats, respectively); *p < 0.003, *p < 0.05, *p < 0.0003 versus control (ipsilateral cortex or hippocampus from control rats, respectively).

Measurements of levels of ascorbate radicals in brain after TBI

Based on the results of our in vitro model experiments we concluded that generation of ascorbate radicals is likely to be detectable in the brain after TBI. Indeed, we found that relatively large ascorbate radical signals were detectable in the initial EPR spectra of brain homogenates from traumatized animals, and the magnitude of the signals continuously increased in the course of incubation (Fig. 6). In control preparations, the magnitude of ascorbate radical signals was much smaller, and it increased over the initial 5 min, after which it remained unchanged during the period of the incubation (Fig. 6). These data confirm that the immediate effect of TBI is manifest as a marked oxidative stress and that ascorbate is the major antioxidant responding to the stress.

DISCUSSION

Biomarkers of oxidative stress in TBI

The brain consumes a disproportionate amount of the body’s oxygen as it derives its energy almost exclusively from oxidative metabolism of the mitochondrial respiratory chain (Coyle and Puttfarcken, 1993) and utilizes it for constitutive oxygenase and oxidase activities such as PG synthase, lipoygenase, heme oxygenase, and tyrosine hydroxylase (Raju et al., 1997; Kim et al., 1999; O’Banion and Olschowska, 1999). Enhanced oxidative stress associated with increased production of free radicals has been implicated in TBI-induced disruption of neuronal homeostasis (Demediuk et al., 1985; Ikeda and Long, 1990; Sen et al., 1994; Awasthi et al., 1997; Inci et al., 1998). Detailed quantitative assessments of bio-markers of oxidative stress as well as depletion of antioxidant reserves by TBI, to the best of our knowledge, have not been con-

TABLE 2. Effect of the COX-2 inhibitor SC 58125 on water-soluble antioxidant reserves in ipsilateral areas of brain cortex and hippocampus after TBI

<table>
<thead>
<tr>
<th>Antioxidant compound (nmol/mg of protein)</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>GSH</th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated (6)</td>
<td>53.1 ± 1.7</td>
<td>53.4 ± 5.0</td>
<td>47.2 ± 1.6</td>
<td>42.7 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>TBI (6)</td>
<td>27.5 ± 1.1a</td>
<td>30.9 ± 2.4a</td>
<td>30.2 ± 1.0a</td>
<td>29.7 ± 2.0a</td>
<td></td>
</tr>
<tr>
<td>TBI + SC 58125 (3)</td>
<td>47.1 ± 2.2a</td>
<td>51.3 ± 3.8b</td>
<td>48.5 ± 1.8a</td>
<td>42.7 ± 2.6a</td>
<td></td>
</tr>
</tbody>
</table>

Rats were given the COX-2 inhibitor SC 58125 at a dose of 30 mg/kg body weight, p.o., 1 h before TBI. The rats were decapitated 24 h after TBI. Water-soluble antioxidant reserves in ipsilateral brain cortex homogenates from sham-operated rats and rats 24 h after TBI were determined by EPR spectral monitoring of tyrosinase-induced oxidation of VP-16 (0.5 mM) to its phenoxyl radical as described in Materials and Methods. Data are mean ± SEM values.

*a p < 0.05 versus sham-operated; *p < 0.02, *p < 0.002, *p < 0.05 versus TBI.
FIG. 5. EPR spectra of ipsilateral brain cortex homogenates. A: EPR spectra of ipsilateral cortex homogenates obtained from DMPO-pre-treated rats at different intervals after TBI. Trace a, sham-operated; trace b, 1.5 h after TBI; trace c, 4 h after TBI; and trace d, 6 h after TBI. DMPO was injected intravenously into rats at a dose of 25 mmol/kg of body weight 5 min before TBI. Brain cortex homogenates were prepared in 40 mM Tris–HCl buffer (pH 7.4) immediately after decapitation. EPR spectra of brain homogenates (2.5 mg of protein/ml) were recorded (receiver gain ×4,000; time constant = 0.03 s) at different time points after TBI. Trace e, the same as trace b plus Fe2+ (100 μM) and H2O2 (1.0 mM); trace f, the same as trace b plus DMPO (200 mM); trace g, 4 h after TBI; and trace h, 6 h after TBI. DMPO-closed radicals or GS• radicals at any of the indicated intervals after TBI were observed. In all cases, however, a pronounced signal of ascorbate radical was detectable in the EPR spectra. B: EPR spectra obtained from ipsilateral brain cortex homogenates incubated in vitro in the presence of an •OH radical-generating system (Fe2+/H2O2 and ascorbate oxidase). Trace a, homogenate prepared from rat brain cortex (2.5 mg of protein/ml, 1.5 h after TBI) in 50 mM disodium phosphate buffer (pH 7.4) immediately after decapitation; trace b, brain cortex homogenate (2.5 mg of protein/ml, 1.5 h after TBI) prepared from DMPO-treated rat (as described above) in 50 mM disodium phosphate buffer (pH 7.4) immediately after decapitation; trace c, the same as for trace b plus Fe2+ (100 μM) and H2O2 (1.0 mM); trace d, the same as trace c plus DMPO (200 mM); trace e, the same as trace b plus treatment with ascorbate oxidase (2.5 U) for 20 min at 25°C; and trace f, the same as trace e plus Fe2+ (100 μM), H2O2 (1.0 mM), and DMPO (200 mM). EPR spectra of brain homogenates were recorded (receiver gain ×2,000; time constant = 0.1 s) as described in Materials and Methods.

The data obtained in this study show that antioxidant reserves were seriously compromised in ipsilateral cortex homogenates, which cannot provide any quantitative or even semiquantitative information due to well-known limitations of the procedure for in vivo measurements (reviewed by Kagan, 1988). We used two different biomarkers for assessing LPO in the brain: 8-epi-PGF2α and fluorescent LPO end products. Although 8-epi-PGF2α can be formed as a result of cyclooxygenase-catalyzed oxidation of arachidonic acid (Pratico et al., 1995), the enzyme does not attack arachidonyl residues of phospholipids. Therefore, we chose to determine generation of phospholipid-derived 8-epi-PGF2α (see Materials and Methods). Under these conditions, 8-epi-PGF2α is a unique product of nonenzymatic free radical peroxidation of phospholipids and is considered to be one of the most reliable biomarkers of LPO in vivo. We found that in the brain the content of 8-epi-PGF2α peaked at 6 h following TBI and significantly decreased thereafter (at 24 h) but still remained higher than in the control (brain of sham-operated rats). The recovery from the maximal accumulation of 8-epi-PGF2α may reflect metabolic conversions of the product, i.e., its β-oxidation (Chiabrando et al., 1999). In contrast, fluorescent end products of LPO showed continuous accumulation after TBI. This corresponds with the polymeric nature of these products whose accumulation during aging (Dillard and Tappel, 1984) indicates that their metabolism seems to be unlikely in neuronal cells. Significantly decreased levels of protein SH groups were found at both 24 and 72 h, indicating that protein cysteines are very sensitive to TBI-induced oxidation and remain oxidized despite the recovery in antioxidant reserves at 72 h (vide infra).

Antioxidant reserves in TBI

The data obtained in this study show that antioxidant reserves were seriously compromised in ipsilateral cor-
text and hippocampus at 6 and 24 h after TBI but recovered at 72 h following TBI. Similar trends but less pronounced changes were found in samples in contralateral areas of the brain (Fig. 3 and Table 1). These results are in keeping with the histopathological evidence of injury in contralateral hemisphere that is present but much less severe than that in ipsilateral areas of the brain in this model (Dixon et al., 1991). This uniform time course of antioxidant reserves was universally observed for all the different assays of antioxidants used in the study—antioxidant reserves, ascorbate, and GSH—with one exception, vitamin E. The latter can be explained by a very effective system of vitamin E recycling by ascorbate and ascorbate/GSH-dependent pathways (Packer et al., 1979; Reddy et al., 1982) operating in the brain. The recycling mechanism maintains vitamin E levels by reducing its radical at the expense of ascorbate/GSH consumption (Kagan et al., 1992). This reaction is dependent on the concentration of these antioxidants and/or the enzymes that maintain them in their reduced form (Reddy et al., 1982). Our results are consistent with the action of the water-soluble antioxidants ascorbate and GSH to reduce vitamin E radicals so as to sustain vitamin E levels in brain cortex and hippocampus under traumatic conditions. It is interesting that transient elevation of extracellular ascorbate levels after cortical compression/contusion trauma was detected in microdialysates from the cerebral cortex in rats (Hillered et al., 1990).

Because antioxidant defenses and biomarkers of oxidative stress fully recovered 72 h after TBI, it is likely that these unregulated oxidation pathways did not develop to such an extent so as to result in irreversible changes.

**Effect of the COX-2 inhibitor SC 58125**

Based on our findings that ascorbate and GSH levels are important indicators of antioxidant status in TBI, we studied the effects of a selective COX-2 inhibitor, SC 58125, on the content of these antioxidants in the brain of rats after TBI. We found that the inhibitor was able to prevent completely loss of ascorbate and GSH at 24 h after TBI. This infers that COX-2 is involved in oxidation reactions responsible for generating radicals in brain in response to TBI. Further studies are required to identify what the particular function of the enzyme is in this process and whether inhibition of enzymatic activity materially affects outcomes of TBI.

**Free radical intermediates in TBI**

The role of free radicals in the pathophysiology of TBI has been the subject of several studies in recent years (Ikeda and Long, 1990; Hall et al., 1993; Mori et al., 1994; Sen et al., 1994; Awasthi et al., 1997). In these investigations, attempts to demonstrate the production of free radicals following TBI have been made using chemiluminescence, salicylate trapping, cytochrome c-covered electrodes, nitro blue tetrazolium reduction, and EPR methods (Kontos and Povlishock, 1986; Wilson et al., 1995; Awasthi et al., 1997; Nishio et al., 1997). Efforts to quantitate radicals that are generated by TBI were not particularly successful despite the fact that enhanced free radical activity has been found (Kontos and Povlishock, 1986; Wilson et al., 1995; Awasthi et al., 1997; Nishio et al., 1997). Protective effects of spin traps against TBI provide strong but indirect evidence for the involvement of free radicals in brain injury as the protection may be due not (only) to radical scavenging but through independent pathways, i.e., via amplified production of antiinflammatory cytokines (Katake et al., 1999).

The results of the present study as well as previously published data (Kihara et al., 1995; Awasthi et al., 1997) unambiguously demonstrate that enhanced formation of ascorbate radicals is a characteristic feature of TBI. In addition, our model experiments with brain homogenates showed that depletion of ascorbate is a prerequisite for detection of oxygen radicals by spin trapping. Together, our results suggest that ascorbate is the primary antioxidant essential for protection of the brain against TBI-induced oxidative stress. Hence, monitoring ascorbate depletion and ascorbate radical production in the brain during oxidative insult may be a valuable method for assessment of antioxidant status and degree of oxidative damage.

**Time course of oxidative stress after TBI**

Some earlier studies that have relied on less quantitative or specific measures of oxidative stress have concluded that the generation of free radicals occurs very early after TBI. For example, Hall et al. (1993) found that OH radicals detected by salicylate trapping occurred exclusively within the first 30 min after TBI. LPO detected by malondialdehyde also peaks within 4 h after injury (Hsiang et al., 1997). The current results suggest that oxidative stress continues for at least 24 h after TBI. Ascorbate radicals are detected in traumatized tissue 6 h after TBI, antioxidants, including ascorbate, are depleted, and there is a progressive accumulation of fluorescent end products of LPO.

**Role of COX-2 activity in producing oxidative stress after TBI**

Previous studies have shown that COX-2 expression is induced after TBI in the controlled cortical impact model (Dash et al., 2000). Furthermore, it is known that COX-2 activity can contribute to oxidative stress in various pathological conditions. The current results show that significant decreases in brain ascorbate and GSH concentrations occur after TBI, concomitant with the formation of ascorbate radicals and other indices of oxidative stress. The consumption of both ascorbate and GSH is prevented by treatment with the selective COX-2 inhibitor, SC 58125. These results suggest that COX-2 activity may be a significant source of oxidative stress after TBI. Nogawa et al. (1998) have shown that COX-2 inhibitors decrease ischemic injury in wild-type mice but not in inducible nitric oxide synthase null mice. These results suggest that these two inflammatory mediators—
prostanoids and nitric oxide—may work synergistically to exacerbate injury in brain, perhaps through the formation of peroxynitrite. Further work is needed to determine the mechanism by which COX-2 exacerbates oxidative stress and brain injury.

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Oxidative stress in traumatic brain injury


