Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia
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Glutamate Release and Free Radical Production Following Brain Injury: Effects of Posttraumatic Hypothermia

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Abstract: Posttraumatic hypothermia reduces the extent of neuronal damage in remote cortical and subcortical structures following traumatic brain injury (TBI). We evaluated whether excessive extracellular release of glutamate and generation of hydroxyl radicals are associated with remote traumatic injury, and whether posttraumatic hypothermia modulates these processes. Lateral fluid percussion was used to induce TBI in rats. The salicylate-trapping method was used in conjunction with microdialysis and HPLC to detect hydroxyl radicals by measurement of the stable adducts 2,3- and 2,5-dihydroxybenzoic acid (DHBA). Extracellular glutamate was measured from the same samples. Following trauma, brain temperature was maintained for 3 h at either 37 or 30°C. Sham-trauma animals were treated in an identical manner. In the normothermic group, TBI induced significant elevations in 2,3-DHBA (3.3-fold, $p < 0.01$), 2,5-DHBA (2.5-fold, $p < 0.01$), and glutamate (2.8-fold, $p < 0.01$) compared with controls. The levels of 2,3-DHBA and glutamate remained high for approximately 1 h after trauma, whereas levels of 2,5-DHBA remained high for the entire sampling period (4 h). Linear regression analysis revealed a significant positive correlation between integrated 2,3-DHBA and glutamate concentrations ($p < 0.05$). Posttraumatic hypothermia resulted in suppression of both 2,3- and 2,5-DHBA elevations and glutamate release. The present data indicate that TBI is followed by prompt increases in both glutamate release and hydroxyl radical production from cortical regions adjacent to the impact site. The magnitude of glutamate release is correlated with the extent of the hydroxyl radical adduct, raising the possibility that the two responses are associated. Posttraumatic hypothermia blunts both responses, suggesting a mechanism by which hypothermia confers protection following TBI. **Key Words:** Trauma—Glutamate—Hydroxyl radicals—Microdialysis—Hypothermia. *J. Neurochem.* **65,** 1704–1711 (1995).

Experimental models of traumatic brain injury (TBI) have been developed to elucidate mechanisms responsible for posttraumatic neuronal damage. We have recently characterized the experimental condition under which fluid percussion brain injury leads to neuronal damage within ipsilateral cortical regions lateral and remote from the impact site (Dietrich et al., 1994*a*). This damage was combined with contusion at the gray–white interface underlying the injured cortical area. It is of interest that hypothermia induced after trauma significantly reduced the extent of the histopathological damage. These results suggest that the pathomechanisms involved in the development of neuronal damage following TBI are temperature sensitive.

Among the mechanisms involved in TBI, excitotoxicity and oxygen free radicals play a central role. Excitotoxicity has been implicated by studies demonstrating massive release of excitatory amino acids at the trauma site (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990; Palmer et al., 1993*a,b*), and by the cytoprotective action of both competitive and noncompetitive NMDA receptor antagonists (Faden et al., 1989; McIntosh et al., 1989; Smith et al., 1993; Hicks et al., 1994), and an α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor blocker (Benert et al., 1992). A role for oxygen radicals in the acute pathophysiology of TBI has been suggested by studies showing that free radical scavengers, such as superoxide dismutase (Chan et al., 1987; Muizellar et al., 1993), and lipid antioxidants, including methylprednisolone (Hall, 1985) and tirilazad mesylate (Hall et al., 1991, 1993), attenuate posttraumatic pathophysiology and/or promote survival and recovery in experimental TBI.

In models of global ischemia, brain hypothermia has been shown to attenuate histopathological damage and to affect several ischemic processes (for review, see

Received February 15, 1995; revised manuscript received April 20, 1995; accepted May 4, 1995.

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Abbreviations used: DHBA, dihydroxybenzoic acid; TBI, traumatic brain injury; TBT, *tert*-butylthiol.

Globus et al., 1992), including inhibition of the extracellular surge in glutamate during the ischemic insult (Busto et al., 1989) and reduction of the amount of hydroxyl radical generation during reperfusion (Globus et al., 1995). In the present study, we evaluated whether excessive extracellular release of glutamate and generation of hydroxyl radicals are associated with injury in regions adjacent to the impact site and whether the protective effect of posttraumatic hypothermia involves modulation of these processes. A portion of this work has been reported in preliminary form (Ginsberg et al., 1995).

MATERIALS AND METHODS

General animal preparation

All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Care Committee. Fasted male Sprague–Dawley rats, weighing 250–300 g, were initially anesthetized with Equitensin (a mixture of nembutal, propylene glycol, ethanol, $MgSO_4$, and chloral hydrate). Intratracheal intubation was performed and rats were placed in a stereotaxic frame. A 4.8-mm craniotomy was made underlying the right parietal cortex 3.8 mm posterior to bregma and 2.5 mm lateral to the midline. A plastic injury tube was placed over the exposed dura and bonded by adhesive. Dental acrylic was then poured around the injury tube. After the acrylic had hardened, the injury tube was plugged with a Gelfoam sponge. For the microdialysis experiment, a guide cannula was affixed to the skull over the parietal cortex (coordinates of 5.0 mm posterior, 5.5 mm lateral to bregma, and 2.0 mm ventral to the dura). The scalp was then closed surgically and the animal was returned to its home cage and allowed to recover overnight.

Induction of TBI

A fluid percussion device was used to produce brain trauma (Dixon et al., 1987). This device consisted of a saline-filled Plexiglas cylindrical reservoir bent at one end by a rubber-covered piston; the opposite end was fitted with a transducer housing an injury screw adapted for the rat's skull. The metal screw was firmly connected to the plastic injury tube of the intubated, anesthetized rat (70% nitric oxide, 1.5% halothane, and the balance of oxygen), and injury was induced. Rats underwent mild-to-moderate head injury ranging from 1.7 to 2.4 atm. Brain temperature was monitored indirectly via a thermocouple probe inserted into the temporalis muscle and maintained at 37°C. As shown previously, temporalis muscle temperature is a reliable indicator of brain temperature following trauma (Jiang et al., 1991). Rectal temperature was also maintained at 37°C prior to and throughout the 3-h posttrauma monitoring period by a heating lamp placed over the animals. Three groups of animals were studied: pooled control nontraumatized rats (37°C, $n = 3$; 30°C, $n = 3$) and animals undergoing TBI followed by 3 h of normothermia (37°C, $n = 8$) or hypothermia (30°C, $n = 5$). In animals in which posttraumatic brain temperature was lowered, cold air was blown directly onto the skull. In these studies, posttraumatic brain temperature was artificially reduced to 30°C starting 3 min after the traumatic insult and maintained there for 3 h. Moderate posttraumatic hypothermia was achieved in all rats within 5 min after trauma.

Microdialysis procedure

On the day of the study, a microdialysis probe with a 2-mm tip (Carnegie Medicin) was inserted through the cannula into the parietal cortex and perfused with modified Ringer's solution at a flow rate of 2 μl/min by means of a microinfusion pump (Carnegie Medicin). Sodium salicylate, 100 mg/kg, i.p., was administered immediately thereafter, followed by a 2-h waiting period before collection of perfusate samples. Following collection of baseline samples, the probe was withdrawn during the administration of the traumatic insult, then reintroduced for sampling. Microdialysis sampling was performed at 10-min intervals up to 4 h following trauma. All samples were collected in an ice bath and then frozen and kept at −20°C until analysis to avoid the effect of freeze-drying due to the small volumes collected. The location of the microdialysis probe was verified histologically in each animal at the end of the experiment. Sham-trauma animals were treated in an identical manner except for the traumatic impact.

Detection of hydroxyl radicals by the salicylate-trapping method

For the measurements of hydroxyl radical production, we utilized salicylate, a relatively nontoxic but highly effective hydroxyl radical trap, which upon scavenging ·OH forms the stable 2,3- and 2,5-dihydroxybenzoic acid (DHBA) (Floyd et al., 1984, 1986). Although 2,5-DHBA may be generated via the cytochrome P-450 system, 2,3-DHBA is regarded as being rather specific for hydroxyl radical production (Halliwell et al., 1991; Ingelman-Sundberg et al., 1991).

The 2,3- and 2,5-DHBA adducts were analyzed in microdialysis samples by an HPLC system (Bioanalytical Systems) consisting of a solvent delivery module (BAS PM-80) that had been customized with two amperometric detectors (BAS-LC-4C), a dual glassy carbon electrode (BAS MF-100), and an Ag/AgCl reference electrode housed in a preheater module (BAS CC-5). The working electrodes were set at +750 mV against the Ag/AgCl electrode. Each amperometric detector was set to a different gain in order to obtain a chromatogram with high sensitivity at low sample concentrations and low sensitivity at high sample concentrations. The separation of different compounds was accomplished by an analytical microbore column C18 5 μm (150 × 1 mm) (BAS Sepstick). The column was used at room temperature with a mobile phase of the following composition: 0.027 m*M* EDTA, 14.7 m*M* NaH_2PO_4, 30 m*M* sodium citrate, 10 m*M* diethylamine HCl, pH 3.1, and 476 mg of octanesulfonic acid to a total volume of 1 L. Thirty milliliters of acetonitrile and 15 ml of tetrahydrofuran were then added. The flow rate was 1 ml/min, and a back-pressure was created with ODS 3 μm (100 × 3.2 mm) column. The system pressure was approximately 3,000 psi. Calibration curves were run daily using 5 μl of four different concentrations of 2,3- and 2,5-DHBA standards.

Extracellular glutamate measurements

Analysis of glutamate concentration in the perfusate was performed by HPLC with electrochemical detection following precolumn derivatization. A two-step derivatization pro-

cess was utilized (Allison et al., 1984). In the first step, a combination of *o*-phthalaldehyde and *tert*-butylthiol (TBT) was used for the formation of isoindole. The substitution of TBT for the more commonly used 2-mercaptoethanol improves the derivative's stability. In order to scavenge the leftover TBT, iodoacetamide was added in a second step. Both the derivatization and the injection of the samples were performed under completely automatic control using the BAS/CMA 200 refrigerated microsampler (Bioanalytical Systems). All work was done on a BAS 200 liquid chromatograph equipped for gradient operation and amperometric detection (Bioanalytical Systems). Amino acids were separated on a microbore column C-18 sepstick with 3-μm particle size (Bioanalytical Systems). The glassy carbon electrode was maintained at +0.7 V versus Ag/AgCl. The mobile phases consisted of dimethylacetamide, methanol, and sodium acetate mixtures (solvent A, 7.0:24.0:69.0, pH 6.8; solvent B, 20:22.5:7.5, pH 8.0, by volume). The gradient profile for this method is based on simple linear changes in both solvents. This method provides good resolution of the common amino acids with a run-time of roughly 30 min. A sharp solvent ramp is used to strip the column after the leucine peak. From peak heights, dialysate concentrations of glutamate were computed by the use of external standards.

Statistical analysis

DHBAs and glutamate levels in rats studied under various conditions (control and trauma) and at two levels of post-traumatic brain temperature (30 and 37°C) were analyzed by means of SAS General Linear Models procedures (SAS Institute, Inc.). These included one- and two-way ANOVA and repeated-measures ANOVA. Posthoc tests included the Bonferroni procedure for multiple intergroup comparisons. The criterion for statistical significance was $p < 0.05$. Linear regression analysis was used to compare the relationship between the amounts of glutamate and DHBAs released in individual animals.

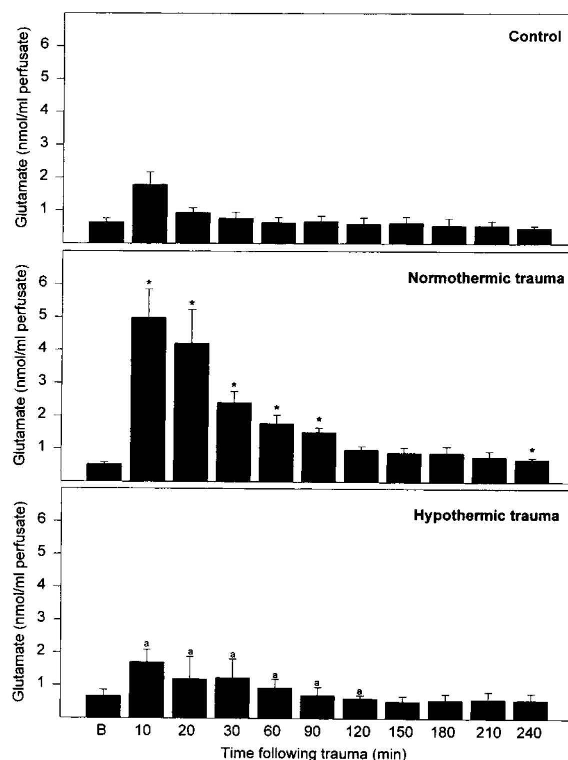

FIG. 1. Levels of glutamate (means ± SEM) in microdialysis perfusate of nontraumatized rats and in animals undergoing fluid percussion injury followed by 3 h of either normothermia (37°C) or hypothermia (30°C). *Significantly different from the corresponding value in nontraumatized rats ($p < 0.05$). [a]Different from the corresponding value in the normothermic group ($p < 0.05$).

RESULTS

The time course of changes in extracellular glutamate in the control group and in the animals undergoing either normothermic or hypothermic trauma is illustrated in Fig. 1. In the control group, there was a slight increase in extracellular glutamate when the microdialysis probe was reinserted. This reflects mass changes in brain tissue following trauma, precluding reinsertion of the probe through the cannula tract made for baseline sampling. As no significant differences were found between the normothermic and hypothermic control groups, they were pooled into one control group. Compared with control conditions, normothermic trauma led to significant increases in extracellular glutamate levels. Repeated-measures ANOVA revealed significant overall between-subjects effects (control versus trauma) for glutamate ($p = 0.005$). The increases in glutamate were observed between 10 and 90 min, and again, slightly, at 240 min following trauma. The maximal increase in glutamate was evident at the 10-min posttrauma time point and represented a 2.6-fold increase above the corresponding control point and a 10-fold increase above baseline levels. In the posttraumatic hypothermic group, the levels of glutamate did not differ from control levels (not significant by repeated-measures ANOVA). These results indicate that posttraumatic hypothermia effectively suppressed glutamate release.

The temporal changes in the DHBAs in the control group and in animals undergoing normothermic and hypothermic trauma are illustrated in Figs. 2 and 3. Similar to what was observed with glutamate, there was a slight increase in extracellular DHBA level when the microdialysis probe was reinserted. As no significant differences were found between the normothermic and hypothermic control groups, they were pooled into one control group. Normothermic fluid percussion injury induced a significant elevation in both 2,3- and 2,5-DHBA in the ipsilateral neocortex. Repeated-measures ANOVA revealed significant overall between-subject effects (control versus trauma) for both 2,3- and 2,5-DHBA levels ($p = 0.003$ and $p = 0.002$, respectively). The increases in 2,5-DHBA were noted throughout the 240-min observation period, with the highest levels observed at 10 min. This peak was 2.5-fold greater than the corresponding control peak, and

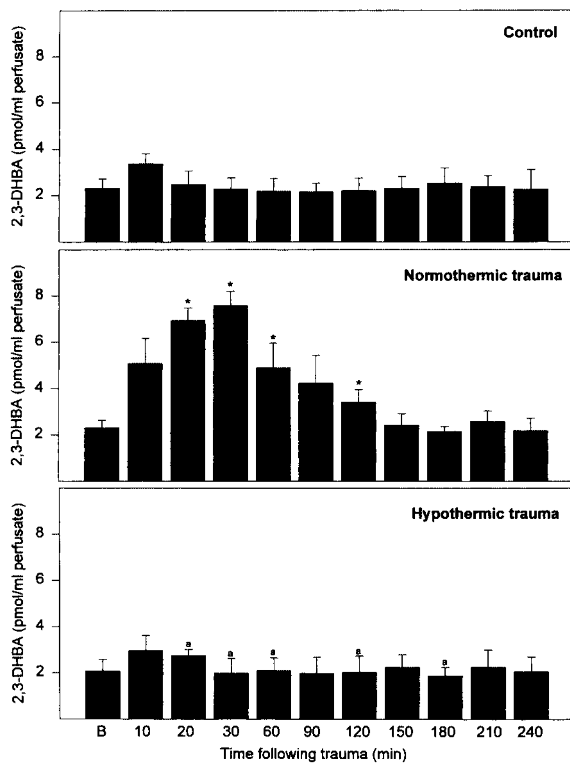

FIG. 2. Levels of 2,3-DHBA (means ± SEM) in microdialysis perfusate of nontraumatized rats and in animals undergoing fluid percussion injury followed by 3 h of either normothermia (37°C) or hypothermia (30°C). *Significantly different from the corresponding value in nontraumatized rats ($p < 0.05$). [a]Different from the corresponding value in the normothermic group ($p < 0.05$).

fourfold above baseline levels. 2,3-DHBA levels increased significantly between 20 and 60 min, and at 120 min posttrauma. The maximum increase in 2,3-DHBA was observed at 30 min and the peak level represented a 3.3-fold increase above the corresponding control level. By contrast, posttraumatic hypothermia resulted in suppression of both 2,3- and 2,5-DHBA elevation. Direct comparison of the two temperature groups by repeated-measures ANOVA revealed a highly significant effect of temperature ($p = 0.001$) for both DHBAs.

To assess the relationship between oxygen radical activity and glutamate release, we analyzed individual 2,3-DHBA, 2,5-DHBA, and glutamate data in the 37°C trauma group (n = 5) and the 30°C trauma group (n = 3) for which all data were available. The individual DHBAs and glutamate levels were first background-corrected by subtracting the average control values at the corresponding experimental points. A linear regression analysis of the resulting data revealed a positive correlation between integrated 2,3-DHBA and glutamate (Fig. 4, $r = 0.743, p = 0.035$). 2,5-DHBA levels were not correlated with glutamate release.

DISCUSSION

Our results indicate that fluid percussion head trauma is followed by a prompt increase in both hydroxyl radical production and glutamate release from cortical regions adjacent to the impact site. The magnitude of glutamate release correlated with the extent of hydroxyl radical production, raising the possibility that the two responses are interconnected. Posttraumatic hypothermia blunted both responses, suggesting a mechanism by which hypothermia confers protection following TBI.

Using microdialysis, other investigators have demonstrated increases in extracellular excitatory amino acids in hippocampal and cortical regions following trauma. The magnitude and the temporal profile of these changes varied among studies. In one study, 90% increases in extracellular levels of glutamate were observed in the hippocampus, peaking at 1–2 min following trauma and returning to baseline levels by 3–4 min (Katayama et al., 1990). In another study, three- to fourfold increases in glutamate were observed, which peaked at 10 min and were sustained for more than 1 h (Faden et al., 1989). The differences between

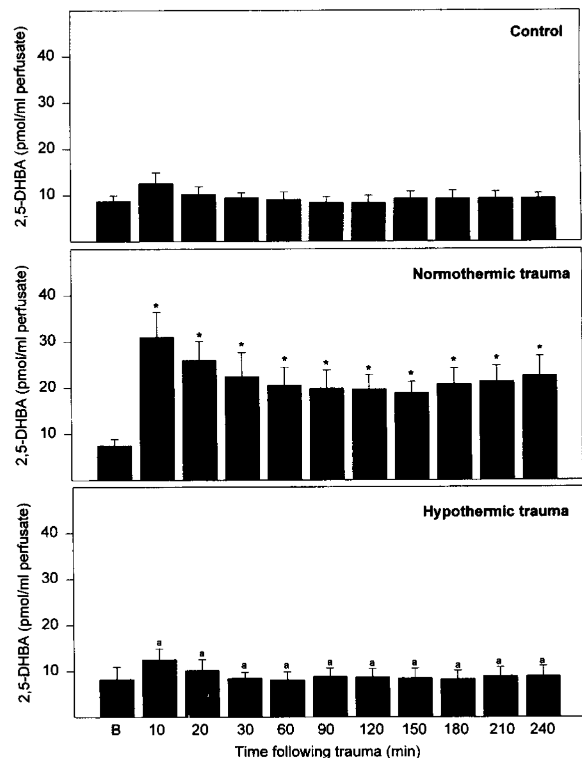

FIG. 3. Levels of 2,5-DHBA (means ± SEM) in microdialysis perfusate of nontraumatized rats and in animals undergoing fluid percussion injury followed by 3 h of either normothermia (37°C) or hypothermia (30°C). *Significantly different from the corresponding value in nontraumatized rats ($p < 0.05$). [a]Different from the corresponding value in the normothermic group ($p < 0.05$).

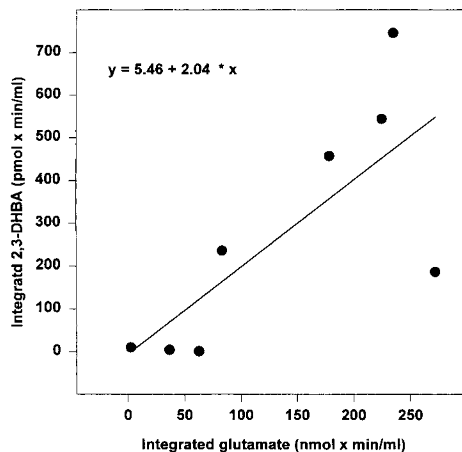

FIG. 4. Linear regression of the integrated 2,3-DHBA levels and the integrated glutamate values following trauma. Increased hydroxyl radical production following trauma was associated with higher levels of extracellular glutamate ($p = 0.035$, correlation coefficient = 0.743).

the two studies may be due to the fact that in the second study the dialysis probe was left in the brain at the impact site and was likely to have increased damage to brain tissue surrounding the probe. In the cortex, the magnitude of changes in extracellular glutamate following TBI was also reported to be greatly variable. Eight- to 13-fold increases were reported in one study (Nilsson et al., 1990), and 81- to 100-fold increases in another (Palmer et al., 1993*a,b*). In both studies, levels of glutamate peaked within 10 min and returned to baseline by 20–30 min. However, these large increases in glutamate were sampled from the site of impact. The tissue surrounding the site of contusion was severely injured, with secondary ischemic changes contributing to the surge in extracellular glutamate. The modest increases in glutamate levels reported in our study may be due to the fact that the microdialysis sampling was done from cortical regions adjacent to, and not from, the injury site itself.

Several mechanisms can be considered to explain the release of glutamate following trauma. Direct mechanical impact can cause depolarization associated with transient increases in extracellular potassium (Katayama et al., 1990). Thus, depolarization may be a triggering event responsible for the release of glutamate. Energy depletion and secondary blood flow changes have been reported following trauma (Nilsson and Nordstrom, 1977; Faden et al., 1989; Yamakami and McIntosh, 1989). Increases in extracellular glutamate have been described in experimental models of ischemia (Benveniste et al., 1984; Globus et al., 1988, 1991). Thus, the surge of extracellular glutamate following trauma could be related in part to secondary ischemic changes. An additional process that may contribute to the increases observed in extracellular glutamate may be related to changes in blood–brain barrier following trauma. Changes in vascular permeability have been described immediately after injury (Cortez et al., 1989; Jiang et al., 1992; Tanno et al., 1992; Dietrich et al., 1994*b*), which could cause entry of excitatory amino acids into the extracellular space from a systemic source. An additional factor that may contribute to the observed elevation in glutamate is that the extracellular space shrinks quickly following concussive brain injury; this may result in a relative increase in concentration of measured substances.

Mild hypothermia has been shown to confer protection following cerebral ischemia (Busto et al., 1987) and traumatic brain injury (Clifton et al., 1991, 1993; Lyeth et al., 1993; Marton et al., 1993; Palmer et al., 1993*a,b;* Dietrich et al., 1994*a*). However, the mechanisms involved in this process remain elusive. Attenuation of the amount of glutamate surge has been suggested as a mechanism for the protective effect of intraischemic hypothermia (Busto et al., 1989). The results from our study firmly establish that posttraumatic increases in extracellular glutamate in cortical regions adjacent to the injury site represent a temperature-sensitive process. Previously, hypothermia was reported to confer protection following TBI without attenuating the rise of interstitial concentrations of aspartate and glutamate (Palmer et al., 1993*a*). However, in that study, measurements of glutamate were done in the central area of contusion, whereas in our study measurements were carried out in a peripheral area adjacent to the injury site. These results suggest that the effects of temperature on glutamate release may depend upon the location of the measurements. In the contusion area, the release of glutamate may be due mainly to the trauma itself, resulting in cell lysis, which could release glutamate from the cytoplasm. This mechanism of glutamate release, which involves tissue destruction, may not be sensitive to temperature. In our study, microdialysis measurements were done in a cortical region that has been shown to display only selective neuronal injury (Dietrich et al., 1994*a*). Thus, the release of glutamate in a brain region adjacent to the injury site does not involve direct mechanical destruction but may be due to one of the processes discussed previously, which may be temperature sensitive. For instance, moderate hypothermia has been shown to reduce blood–brain barrier disruption following TBI (Jiang et al., 1992).

In addition to evidence for a temperature-sensitive surge in glutamate, the results of the present study demonstrate a temperature-dependent increase in hydroxyl radicals following trauma. Earlier studies have demonstrated generation of superoxide radicals in the injured brain using the chemical reduction of nitro blue tetrazolium applied to brain surface through a cranial window (Kontos and Povlishock, 1986). A more recent study, using a spin-trapping agent combined with electron spin resonance detection, has also provided

evidence for free radical release following brain concussion (Sen et al., 1994). In our study, we have used the salicylate-trapping method coupled with microdialysis to provide direct evidence for increased formation of hydroxyl radicals after TBI. Similar changes in tissue levels of 2,5-DHBA following TBI were described recently (Hall et al., 1993; Smith et al., 1994). Our study has expanded upon this earlier study in several ways.

First, in the earlier study, only the changes in 2,5-DHBA were documented. Because it has been suggested that changes in 2,5-DHBA may reflect cytochrome P-450 activity (Halliwell et al., 1991), measurements of only the 2,5-DHBA adduct may be misleading. In contrast, no endogenous enzyme is known to convert salicylate to 2,3-DHBA; therefore, changes in this adduct are considered to be highly specific for hydroxylation of salicylate (Ingelman-Sundberg et al., 1991). In our study, we included measurements of both 2,3- and 2,5-DHBA. The comparable changes in both adducts strongly suggest that they represent hydroxyl radical formation.

Second, our data demonstrate the feasibility of using the salicylate-trapping method in conjunction with microdialysis to detect hydroxyl radical activity following trauma. This approach has been used successfully by other investigators to detect hydroxyl radical activity in the brain following MPTP (Obata and Chiueh, 1992) or NMDA (Hammer et al., 1993) administration. The microdialysis technique enabled us to measure hydroxyl radical activity continuously in the same animal following TBI. The results clearly indicate that hydroxyl radical formation is not a short-lived event but rather a phenomenon that lasts 1–2 h following trauma.

Third, our study is the first to demonstrate that formation of hydroxyl radicals in the brain following trauma is a temperature-dependent process. It is plausible, therefore, that a mechanism by which posttraumatic hypothermia alters histopathological outcome involves modulation of free radical formation.

Our data also demonstrate that the magnitude of glutamate release correlated with the extent of hydroxyl radical production, raising the possibility that the two processes are interrelated in TBI. This conjecture is supported by the results of recent studies suggesting that interaction between excitatory amino acids and oxygen radicals is part of the ischemic injury process. Pellegrini-Giampietro et al. (1990) provided evidence suggesting that oxygen-derived free radicals induce excitatory amino acid release in rat hippocampal slices. They proposed that a vicious cycle exists in which free radicals and excitatory amino acids interact with each other in the propagation of ischemic neuronal damage. Other studies raise the possibility that glutamatergic mechanisms may be a trigger for the hydroxyl radical production. In cultured cerebellar granular cells, superoxide radical was shown to be produced by NMDA receptor activation (Lafon-Cazal et al., 1993). In vivo studies have shown that local administration of glutamate (Boisvert and Schreiber, 1992) or NMDA (Hammer et al., 1993) is associated with formation of hydroxyl radicals in the brain. Further support for the role of free radicals in glutamate toxicity is provided by a study showing that excitotoxicity in neuronal cultures is attenuated by inhibitors of lipid peroxidation (Monyer et al., 1990). Our results, demonstrating a correlation between the glutamate surge and the production of hydroxyl radicals following TBI, suggest that oxygen free radicals and excitatory amino acids may interact with each other in the generation of TBI.

In summary, our data provide evidence for glutamate release and hydroxyl radical production in cortical regions adjacent to the injury site. A significant correlation between the magnitude of glutamate release and the extent of hydroxyl radical production was demonstrated, suggesting a linkage between the two responses. Posttraumatic hypothermia effectively attenuated the rise in glutamate and the generation of the free radicals. A possible mechanism underlying the protective effect of posttraumatic hypothermia is thus suggested.

Acknowledgment: This study was supported by USPHS grants NS-30291, NS-05820, and NS-26784. We are indebted to Elena Martinez for providing expert technical assistance, and to Helen Valkowitz for helping to prepare the manuscript.

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