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N-Acetylaspartate Reduction as a Measure of Injury Severity and Mitochondrial Dysfunction Following Diffuse Traumatic Brain Injury

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ABSTRACT

***N*-Acetylaspartate (NAA) is considered a neuron-specific metabolite and its reduction a marker of neuronal loss. The objective of this study was to evaluate the time course of NAA changes in varying grades of traumatic brain injury (TBI), in concert with the disturbance of energy metabolites (ATP). Since NAA is synthesized by the mitochondria, it was hypothesized that changes in NAA would follow ATP. The impact acceleration model was used to produce three grades of TBI. Sprague-Dawley rats were divided into the following four groups: sham control ($n = 12$); moderate TBI ($n = 36$); severe TBI ($n = 36$); and severe TBI coupled with hypoxia-hypotension ($n = 16$). Animals were sacrificed at different time points ranging from 1 min to 120 h postinjury, and the brain was processed for high-performance liquid chromatography (HPLC) analysis of NAA and ATP. After moderate TBI, NAA reduced gradually by 35% at 6 h and 46% at 15 h, accompanied by a 57% and 45% reduction in ATP. A spontaneous recovery of NAA to 86% of baseline at 120 h was paralleled by a restoration in ATP. In severe TBI, NAA fell suddenly and did not recover, showing critical reduction (60%) at 48 h. ATP was reduced by 70% and also did not recover. Maximum NAA and ATP decrease occurred with secondary insult (80% and 90%, respectively, at 48 h). These data show that, at 48 h post diffuse TBI, reduction of NAA is graded according to the severity of insult. NAA recovers if the degree of injury is moderate and not accompanied by secondary insult. The highly similar time course and correlation between NAA and ATP supports the notion that NAA reduction is related to energetic impairment.**

Key words: energetic metabolism; high-performance liquid chromatography; magnetic resonance spectroscopy; mitochondrial dysfunction; *N*-acetylaspartate; traumatic brain injury

INTRODUCTION

DIFFUSE BRAIN INJURY, defined by the absence of focal mass lesions, is associated with high morbidity rates and considered the most common cause of severe disability and posttraumatic persistent vegetative state (Graham et al., 1983; Kinney and Samuels, 1994). The pathogenesis of diffuse injury is thought to occur as a re-

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sult of shearing or impulsive forces on axons (Adams et al., 1977, 1984, 1989).

Although conventional brain imaging techniques are useful in detecting macroscopic changes, patients with diffuse brain injury often show a radiologically "normal brain" (Eisenberg et al., 1990). At present, there are few practical methods for assessing the severity of this type of injury and its potential for recovery in the head-injured patient. As a result, diffuse brain injury is an underdiagnosed entity with few prognostic elements and its pathophysiology is not fully understood.

Recently, *N*-acetylaspartic acid (NAA) has been identified as an *in vivo* marker of neuronal density, and its reduction is related to neuronal damage and loss in many cerebral disorders (Davie et al., 1997; Ebisu et al., 1994; Harms et al., 1997; Sager et al., 1995, 1999; Shino et al., 1993). Being the most prominent compound detectable with proton magnetic resonance spectroscopy (^{1}HMRS) in the human brain and therefore representing a noninvasively measurable cerebral metabolite, NAA has faced increasing scrutiny in neurotrauma research during the past 5 years (Brooks et al., 2000; Cecil et al., 1998; Choe et al., 1995; Friedman et al., 1998; Ross et al., 1998; Rubin et al., 1997). Reduction of NAA levels following blunt TBI is generally accepted as evidence of both posttraumatic neuronal and axonal damage even when demonstrated in areas of normal appearing cerebral cortex (Friedman et al., 1999; Ricci et al., 1997), hemispheric white matter, and corpus callosum (Cecil et al., 1998; Garnett et al., 2000b; Smith et al., 1998). However the precise role and significance of NAA reduction after TBI is still controversial (Alessandri et al., 2000; Al-Samsam et al., 2000).

The first objective of this study was to investigate the time course of NAA changes in varying grades of diffuse TBI to support the notion that reduction of NAA is proportional to injury severity as recently proposed by Garnett et al. (2000). However, the majority of the aforementioned studies using ^{1}HMRS for NAA quantification have, by necessity, reported results in the form of ratios of peak areas, typically NAA/creatine (Cr) or NAA/choline (Cho). This methodology assumes constancy in the concentration of either Cr or Cho, a condition not necessarily correct after trauma. To obtain absolute metabolite concentrations we measured whole-brain NAA by high-performance liquid chromatography (HPLC) analysis (Tavazzi et al., 2000).

The second objective was to provide evidence that NAA reduction in not solely related to neuronal cell loss, but also might depend on the amply demonstrated energetic impairment due to posttraumatic mitochondrial dysfunction (Ahmed et al., 2000; Fiskum, 2000; Vagnozzi et al., 1999; Verweij et al., 2000; Xiong et al., 1997). The background for this hypothesis is the fact that several studies have furnished strong evidence that NAA reduction can reflect energetic disturbance secondary to mitochondrial dysfunction (Bates et al., 1996; Clark, 1998). To assess energetic status, we synchronously measured the whole-brain ATP concentration.

MATERIALS AND METHODS

Experimental Protocol and Surgical Preparation

All surgical procedures were considered and approved by VCU Institutional Animal Care and Use Committee Regulations, in compliance with NIH standards and guidelines.

One hundred adult Sprague-Dawley rats, weighing 350–400 g (375.5 ± 15.6 g), were randomly divided into the following experimental groups: (1) sham control (n = 12); (2) moderate diffuse traumatic brain injury (n = 36); (3) severe diffuse traumatic brain injury (n = 36); (4) severe diffuse traumatic brain injury combined with 10 min of hypoxia-hypotension (THH-10; n = 16). The rats were initially anesthetized with halothane anesthesia (4%). The animals were then intubated under direct vision and mechanically ventilated through an endotracheal tube with a gas mixture of N_2O (70%), O_2 (30%), and halothane (0.5–1.5%) using a Narcomed ventilator. A femoral artery was cannulated with PE-50 tubing (Becton Dickinson & Company, Parsippany, NJ), for measuring mABP and arterial blood gases. Mean arterial blood pressure was maintained at 100 mm Hg. In animals that did not undergo secondary insult, blood gases were maintained throughout the entire experiment with a pO_2 of 100–130 mm Hg and a pCO_2 of 35–40 mm Hg. The animals' temperature was maintained at 36.5–37.5°C using a rectal temperature probe (YSI, Inc., model 73A, Yellow Springs, OH) and a heating lamp. Following stabilization of blood gases and blood pressure, the animal was positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A midline scalp incision was made, the skin and periosteum were reflected, and the skull was carefully dried. A 1-cm-round stainless steel disk was mounted on the skull by using acrylic super glue positioned midline between bregma and lambda.

Moderate and Severe Diffuse Traumatic Brain Injury

The impact acceleration head injury model (Foda and Marmarou, 1994; Marmarou et al., 1994) was used to produce trauma. A cylindrical column of segmented brass

weighing either 450 or 500 g was dropped through a Plexiglas tube onto the disc fixed to the skull vault of the animal. The severity of the injury was adjusted by varying the mass of the brass weight and/or the height it fell through. According to the biomechanics of this model and the pathophysiological changes previously described in detail (Marmarou et al., 1994), an impact of 500 g from a height of 1 m was used to induce a moderate head injury; impact of 450 g from 2 m was considered a severe head injury. A full description of the methodology of the impact acceleration model has been reported previously (Marmarou et al., 1994).

Briefly, after the bonding agent was dry and the metal disk firmly fixed, the animal was disconnected from the respirator and placed in a prone position on a foam bed of known elastic parameters and secured in place with two belts. The injury was delivered by dropping the chosen weight from the predetermined height. After trauma, the rat was rapidly reconnected to anesthesia and artificially ventilated. Rats that did not survive the impact or suffered skull fractures were excluded from the study. Sham-operated animals underwent the same procedures with the absence of the actual impact.

Trauma Coupled with Secondary Insult

The rationale of adding a secondary insult to this group of animals derives from the clinical setting, where early hypotension and hypoxia has been proven to cause catastrophic effects on the injured brain, and is an important indicator of poor outcome. (Chesnut et al., 1993a,b; Signorini et al., 1999) The method of superimposing a secondary hypoxic-hypotensive insult after trauma was recently developed in our laboratory and reported elsewhere (Yamamoto et al., 1999). Briefly, immediately after the 450-g/2-m trauma, in the group of animals undergoing secondary insult, hypoxia and hypotension were induced by manipulation of anesthesia (Table 1). A reduction of FiO_2 to 12% resulted in hypotension of 35.5 ± 5.3 mm Hg and hypoxia of 44.5 ± 5.1 mm Hg (PaO_2). Additionally, the inspired concentration of halothane was increased by a maximum of 2% in order to blunt the adrenergic response and maintain the stability of the insult. Hypoxia and hypotension were sustained for 10 min, after which anesthesia was returned to pretrauma configuration.

Table 1. Physiological Variables During the Secondary Insult

	Preinsult	*THH-10*[a]	*Postinsult*[b]
MABP	115.5 ± 10.5	35.5 ± 5.5	112.7 ± 3.8
Ph	7.42 ± 0.04	7.35 ± 0.05	7.36 ± 0.04
$PaCO_2$	38.0 ± 3.0	33.0 ± 5.0	36.5 ± 3.5
PaO_2	120.5 ± 10.5	44.5 ± 5.1	130 ± 10.5

[a]Values were obtained during secondary insult.

[b]Values were obtained 1 h postinsult.

Values are expressed as the mean ± standard deviation of four different animals.

THH-10, severe trauma combined with 10 min of hypoxia and hypotension.

Brain Sampling and Tissue Preparation

Animals designated to survive more than 30 min were extubated and the scalp wound sutured. At the end of the desired survival period, rats were again anesthetized and sacrificed. In moderate and severe injury groups, animals ($n = 4$) were sacrificed at the following time points after trauma: 1 min, 10 min, 30 min, 2 h, 6 h, 15 h, 24 h, 48 h, and 120 h. In the THH-10, group animals ($n = 4$) were sacrificed at 2 h, 6 h, 24 h, and 48 h. Sham animals ($n = 4$ for each of the three groups) were sacrificed at 24 h.

In vivo craniectomy was performed, and the whole brain was directly transferred from the skull into liquid nitrogen using a surgical spatula. This procedure not only avoids possible ischemic conditions, but also permits a quicker and more homogeneous cerebral tissue freezing than other commonly used brain processing techniques such as pouring liquid nitrogen *in situ* (Plaschke et al., 1999). Animals considered as shams were exposed to exactly the same experimental manipulations. The frozen brain was then weighed and the cerebral tissue deproteinized (Lazzarino et al., 1989) by homogenization for 60 sec in ice cold 1.2 M perchloric acid ($HClO_4$; 1:10; w:v) at maximum speed using an Ultra-Turrax homogenizer (Janke and Kunkel, IKA-Werk, Staufen, Germany). Perchloric acid homogenate was then centrifuged at 20,190*g* for 15 min at 4°C; the supernatant was saved and the pellet was homogenized again with 2 mL of ice-cold 1.2 M $HClO_4$. After centrifugation, supernatants were combined, neutralized with 5 M potassium perchlorate (K_2CO_3), and immersed for 90 sec in liquid nitrogen in order to optimize K_2CO_3 precipitation. Samples were then centrifuged (20,190*g*) for 10 min at 4°C, supernatants were extracted with chloroform (1:1; v:v; Lazzarino et al., 1991) in order to remove any lipid-soluble materials, they were centrifuged again, and the upper aqueous phase was saved at −80°C. The neutralized chloroform-extracted solution was filtered through a 0.45 μM HV-Millipore filter and then loaded (100 μL) onto the column for HPLC analysis.

High-Performance Liquid Chromatography Analysis

The HPLC apparatus consisted of a Constametric 3500 dual pump system (ThermoQuest Italia, Rodano, Milan, Italy) set at a wavelength of 190–300 nm. Data were acquired and analyzed using ChromQuest software provided by the HPLC manufacturer. Concentrations of ATP were determined on a 50-μL sample by an ion-pairing HPLC method (Lazzarino et al., 1991) using a Kromasil 250 $\times$ 4.6 mm, 5-μ particle size column, provided with its own guard column (Eka Chemicals AB, Bohus, Sweden), and tetrabutylammonium hydroxide as the pairing reagent. Different metabolites were separated as described in detail elsewhere (Lazzarino et al., 1989; Vagnozzi, et al., 1999) ATP was identified, and absolute concentration was determined at 267-nm wavelength, comparing both retention times and absorption spectra of each sample chromatogram with the same parameters of freshly prepared ultrapure standard.

Separation of NAA was obtained isocratically, according to a method recently developed in our laboratories (Tavazzi et al., 2000), by using a mobile phase buffer (Buffer A) with the following composition: 2.8 mM tetrabutylammonium hydroxide, 25 mM KH_2PO_4, 1.25% methanol, pH adjusted to 7.00. The flow rate was 1 mL/min, and the temperature was kept constant at 23°C.

After each chromatographic run of cerebral tissue extracts (20 μL), the column was washed for at least 20 min at a flow rate of 1.3 mL/min with a washing solution (Buffer B) with the following composition: 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.50. Before injecting a new sample, a subsequent column conditioning for 15 min at a flow rate of 1.3 mL/min with the separating buffer A was performed. Assessment of NAA concentration was made at 210-nm wavelength using the same purity criteria and comparison of absorption spectra utilized for the ATP identification.

Statistical Analysis

ATP and NAA levels were expressed as the mean $\pm$ standard deviation of four animals at each time point. For both metabolites, statistical differences between sham-controls and injured animals at different time points, within the same injury severity group, and between the three levels of trauma, was tested by analysis of variances (ANOVA) and posthoc Fisher's PLSD test. The same procedure was used to test the difference between the injury severity groups at 2 h, 6 h, 24 h, and 48 h. The relationship between the time course of NAA and ATP observed in moderate, severe, and THH injury was tested by simple regression and correlation analysis. A 95% confidence level was considered statistically significant.

RESULTS

N-Acetylaspartate and ATP Changes in Moderate Injury

The time courses of NAA and ATP changes are represented in Figure 1. In the control group, the mean level of NAA equaled 9.84 $\pm$ 0.88 μmol/g w.w. Following moderate TBI, NAA fell within minutes, reaching statistical significance after 10 min (7.88 $\pm$ 0.91 μmol/g w.w., $p < 0.01$). A further NAA reduction of 35% of the baseline level was detected at 6 h (6.15 $\pm$ 0.1 μmol/g w.w., $p < 0.0001$), while maximum decrease was evident at 15 h postinjury when NAA reached 46% of the control value (5.34 $\pm$ 1.14 μmol/g w.w., $p < 0.0001$). From this time point up to 5 days postinjury, NAA showed a progressive recovery, reaching 86% of the baseline at 120 h ($p = 0.04$).

The time course of ATP changes mirrored the NAA changes. The mean ATP concentration in control animals equaled 2.41 $\pm$ 0.15 μmol/g w.w. A gradual reduction of ATP started within minutes and reached statistical significance at 2 h postinjury, at which time ATP concentration was diminished by 38% of baseline (1.9 $\pm$ 0.38 μmol/g w.w., $p < 0.005$). The lowest ATP value was found at 6 h postinjury showing a decrease of 57% (1.03 $\pm$ 0.14 μmol/g w.w., $p < 0.0001$). At 15 h postinjury, ATP remained 45% below baseline, despite a trend toward recovery. ATP restoration continued at later time points, reaching 78% of baseline at 48 h (1.88 $\pm$ 0.26 μmol/g w.w., $p < 0.05$), and at 120 h postinjury ATP reduction in injured animals was not significantly different from control animals.

N-Acetylaspartate and ATP Changes in Severe Injury

The time course of NAA and ATP changes are represented in Figure 2. One minute following severe TBI, NAA had fallen by 25% compared with control (7.43 $\pm$ 0.57 μmol/g w.w., $p < 0.0001$). From this time point up to 24 h, there was no further significant reduction in NAA concentrations. In the 24-h group, NAA was significantly higher than in the 15-h group (8.65 $\pm$ 0.92 μmol/g w.w. and 7.18 $\pm$ 0.67 μmol/g w.w., respectively, $p < 0.01$), but was still diminished with respect to the baseline (11% decrease, $p < 0.05$). After this period of apparent partial recovery, a dramatic reduction by a further 48% of the control values occurred at 48 h postinjury (5.13 $\pm$ 0.98

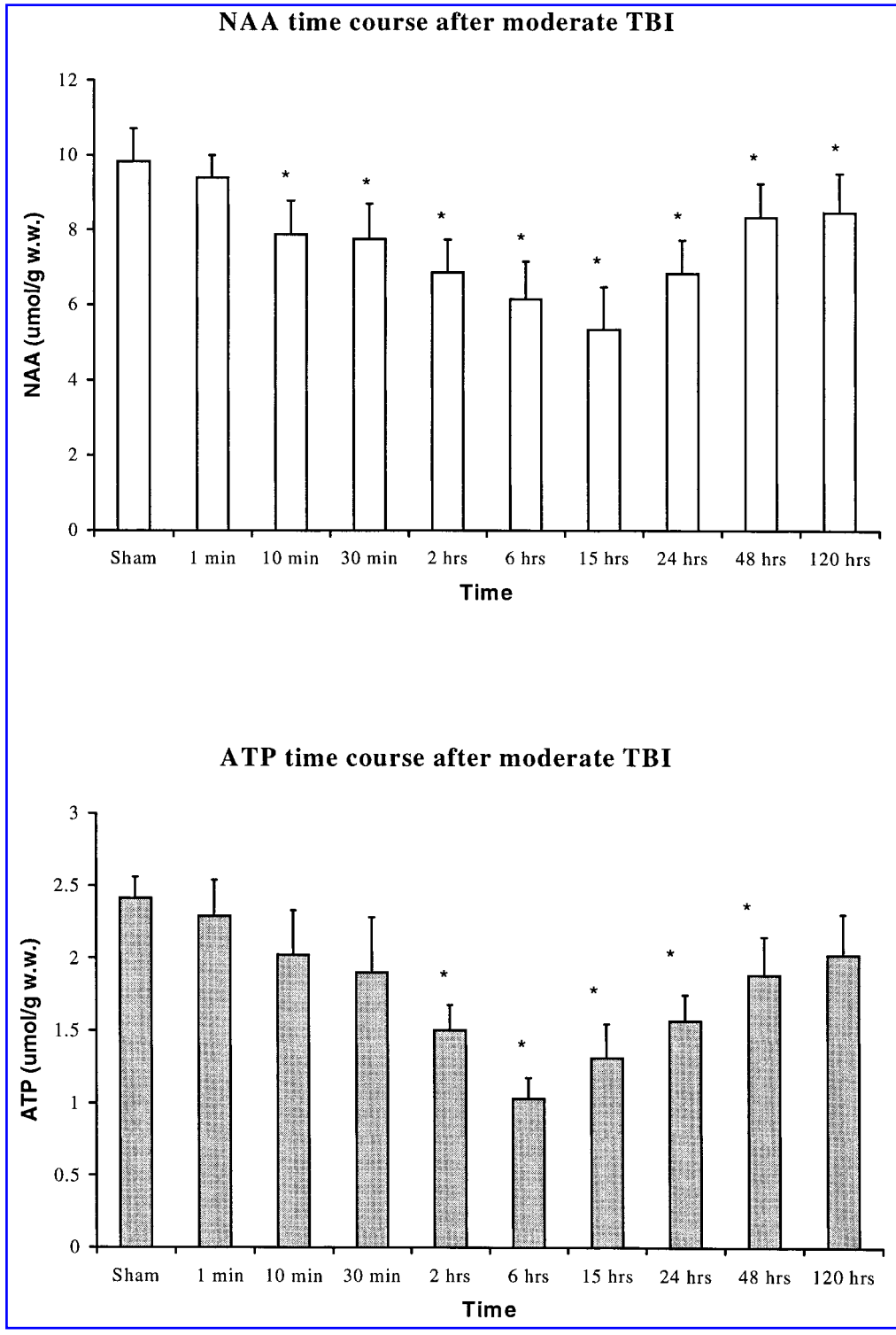

FIG. 1. Concentrations of NAA and ATP determined by HPLC analysis of perchloric acid brain tissue extract. Following moderate diffuse TBI, recovery of NAA is observed, and it is paralleled by restoration of ATP.

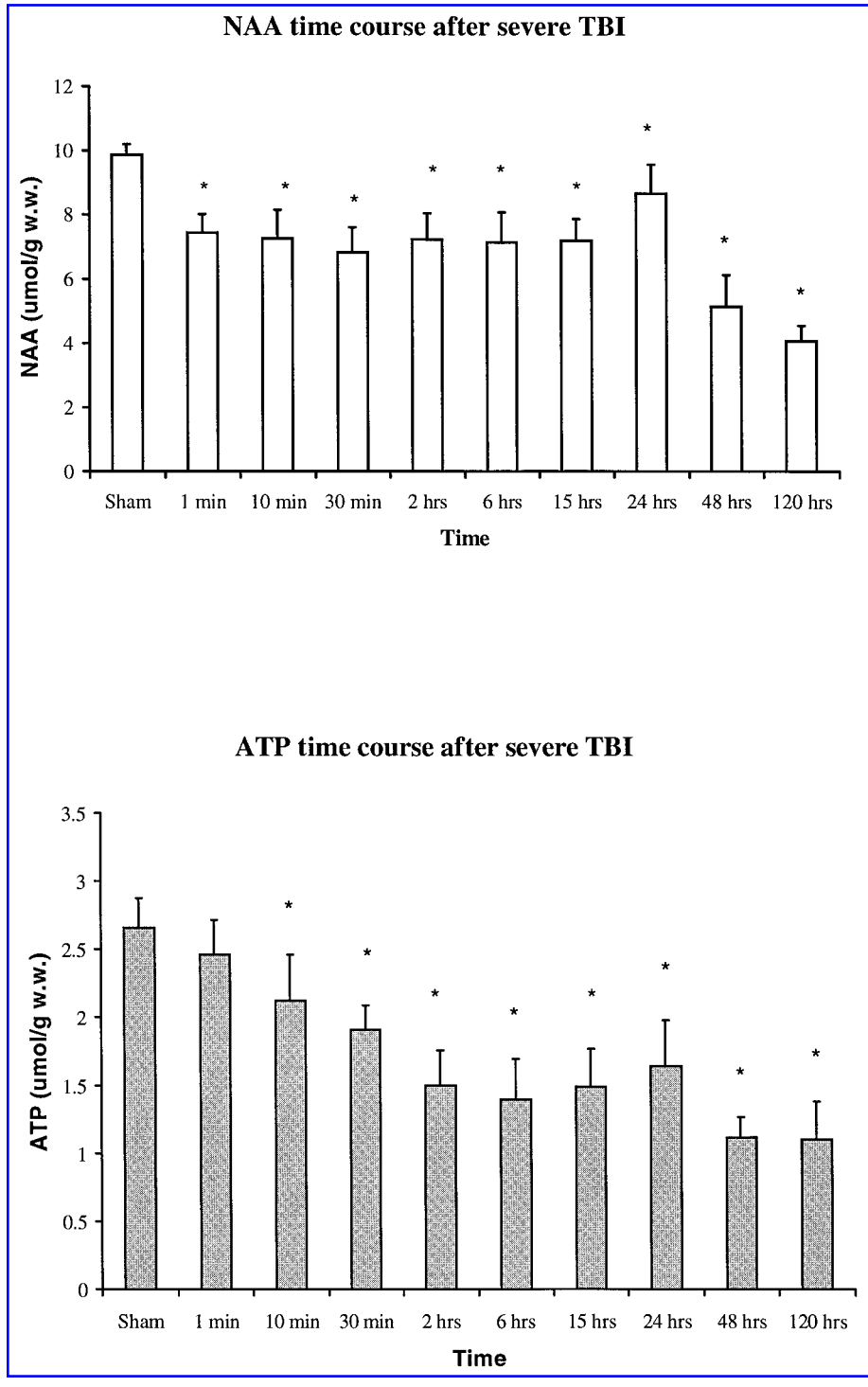

FIG. 2. Concentrations of NAA and ATP after severe diffuse TBI. NAA shows a sudden reduction followed by a plateau. Between 24 and 48 h, maximal NAA decrease occurs with no recovery. The lacking of ATP restoration testifies to the persistent energetic crisis.

μmol/g w.w., $p < 0.0001$), reaching a 59% reduction at 120 h (4.06 $\pm$ 0.47 μmol/g w.w.).

A similar time course was seen in the ATP concentrations. Ten minutes after trauma, a significant reduction of ATP (20% of the control values) was already detectable (2.12 $\pm$ 0.34 μmol/g w.w., $p < 0.01$), and after 30 min a 28% decrease was observed (1.91 $\pm$ 0.17 μmol/g w.w., $p < 0.001$). At 2 h postinjury, ATP had de-

creased by 44% of baseline (1.5 ± 0.26 μmol/g w.w., $p < 0.0001$). This value represented a plateau with no further statistically significant reduction detectable up to 24 h. However, at 24 h postinjury, ATP showed a partial recovery, reaching 62% of baseline values (1.64 ± 0.33 μmol/g w.w., $p < 0.005$). At 48 h, a second dramatic reduction of ATP (58% of the control values) was observed, with no further significant changes occurring up to 5 days postinjury (1.12 ± 0.15 μmol/g w.w., $p < 0.0001$).

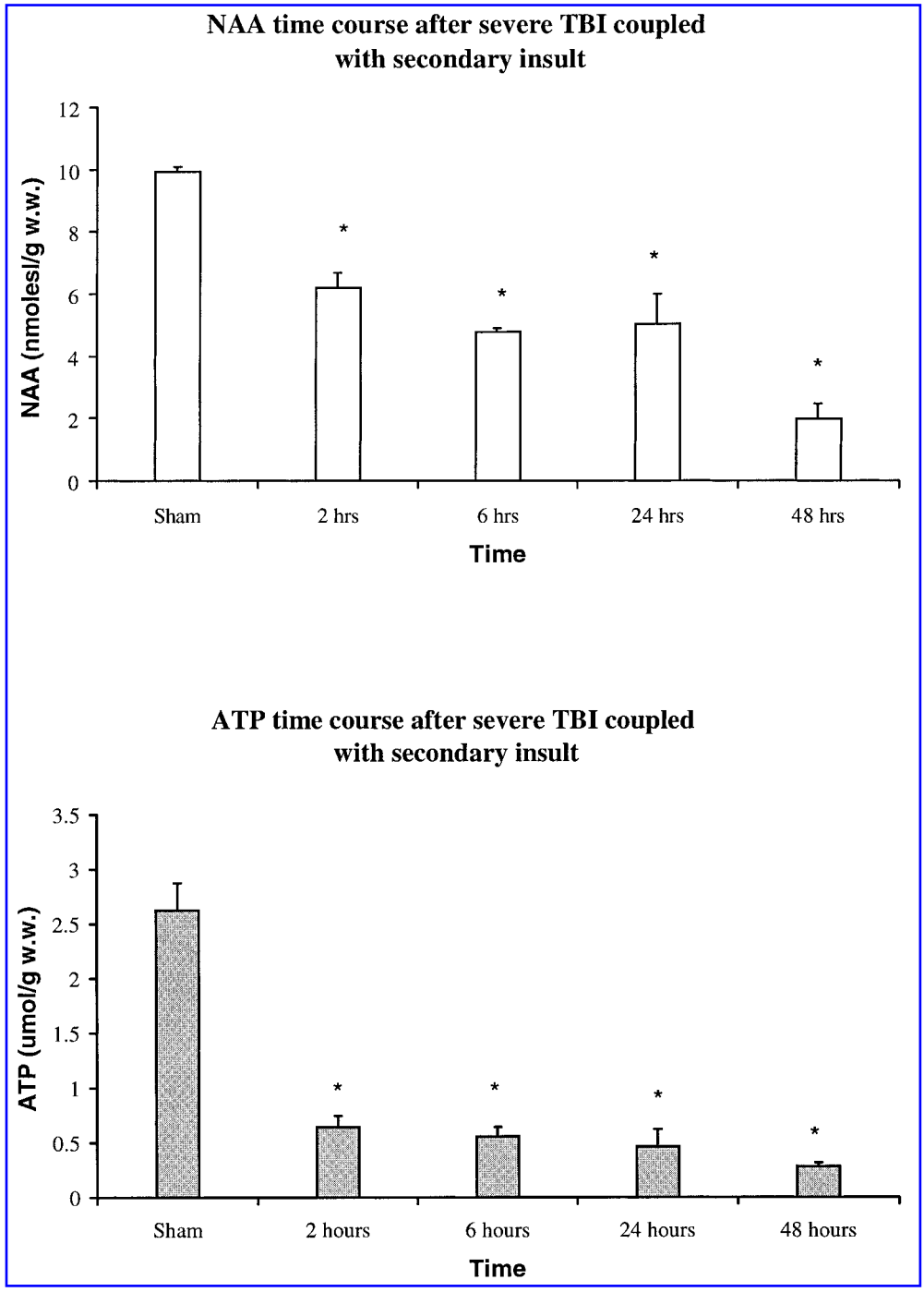

FIG. 3. Concentration of NAA and ATP after severe diffuse TBI, coupled with 10 min of posttraumatic hypoxia-hypotension. Secondary insult strongly reduces NAA concentrations at 48 h. Severe energetic unbalance is testified by a 90% reduction in ATP at this time point.

Severe Head Injury Coupled with Secondary Insult

The time courses of NAA and ATP changes for severe injury with secondary insult are represented in Figure 3. Following hpoxia-hypotension, a sharp and more severe reduction of NAA was observed. At 2 h postinjury, NAA was reduced by 30% compared with control values and by 52% at 6 h (6.18 ± 0.51 μmol/g w.w., $p < 0.0001$, and 4.76 ± 0.13 μmol/g w.w., $p < 0.0001$, respectively). No further statistically significant changes were observed up to 24 h. At 48 h postinjury a dramatic decrease of 81% compared with baseline was observed (1.97 ± 0.5 μmol/g w.w., $p < 0.001$ compared to the 24-h group).

Two hours following injury, ATP already showed a 73% reduction (0.64 ± 0.1 μmol/g w.w.) compared with the control group. After this time, although slight reductions were noticed at 24 h and 48 h postinjury, no further significant changes were detected. However, it is noteworthy that the mean value of ATP at 48 h showed a 90% reduction.

Comparison of Injury Severity and NAA-ATP Reduction

Analysis of the three different levels of trauma at 2 h postinjury showed no statistical difference with respect to NAA reduction. At 6 h and 24 h postinjury, the moderate and severe injury groups did not exhibit differences; only the THH group showed a significant NAA reduction. ATP differences were significant at 2 h but only in THH animals, with a 53% reduction compared to the other trauma groups. At 48 h postinjury, the three levels of injury showed the most significant differences (Fig. 4). Most importantly, both NAA and ATP were restored at later time points in moderate injury. Compared to this "recovering" group, in the severe injury and THH groups, NAA was reduced by 38% and 77%, respectively. ATP exhibited a proportional reduction of 40% and 85% at the same time point.

DISCUSSION

The results of the present study demonstrate that, after diffuse TBI, the reduction of NAA observed at 48 h postinjury is graded according to the severity of insult. Following moderate injury, NAA reduced gradually and recovered spontaneously to reach baseline levels. Severe trauma and severe trauma coupled with secondary insult showed a sudden and progressive NAA fall, which was irreversible up to 5 days. The difference in NAA levels between the three grades of injury was obvious at 48 h, with maximum NAA reduction occurring in the secondary insult group. With regard to our second objective,

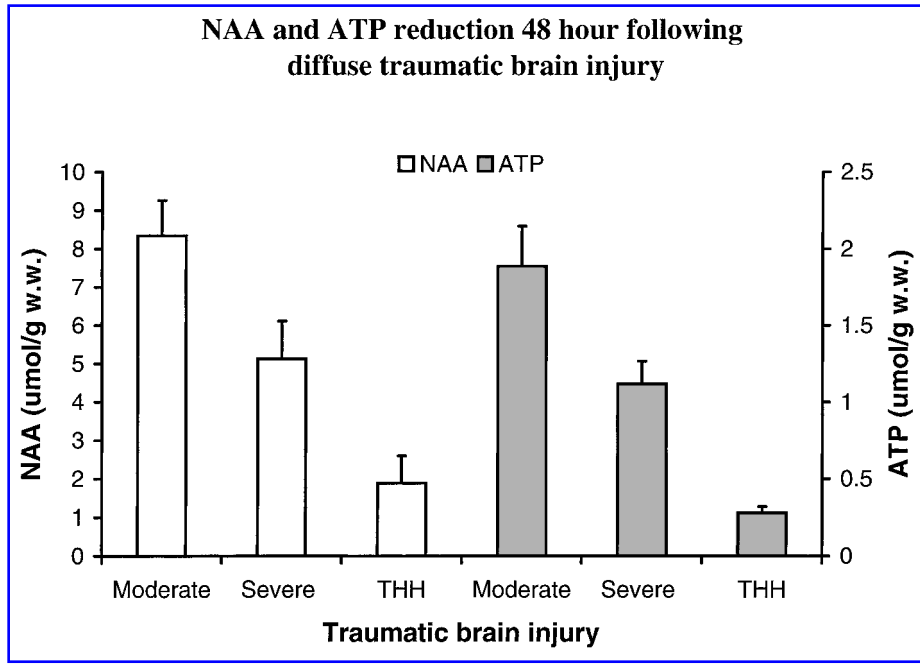

FIG. 4. Concentration of NAA and ATP at 48 h postinjury showing that graded levels of injury correspond to graded biochemical damage. In moderate trauma, ATP and NAA recover. In severe trauma alone and in trauma with secondary insult, reduction of NAA and ATP is proportional to the severity of injury.

the time course of ATP change closely followed the pattern of NAA change. Recovery of ATP coincided with NAA recovery in moderately injured animals, while in severe injuries, with sustained ATP depletion, NAA loss persisted. This provides compelling evidence to support the notion that NAA reduction can serve as an indicator of energetic metabolism impairment.

N-Acetylaspartate Reduction after Traumatic Brain Injury

While the exact function of NAA is as yet uncharacterized, many different roles have been suggested (Birken and Oldendorf, 1989; Clarke et al., 1975; D'Adamo et al., 1968; Mehta and Namboodiri, 1995; Patel and Clark, 1980; Taylor et al., 1995).

In a previous report by Rubin et al. (1997) a significant posttraumatic reduction of NAA was observed in the rodent using a parasagittal fluid percussion model. Using high-resolution ^{1}HMRS, a progressive loss of NAA was documented at 1 h postinjury over a period of 14.5 min. In another ^{1}HMRS study, Smith et al. (1998) described a 20% drop in NAA acutely after diffuse brain injury in the miniature swine model. This reduction was observed in regions of histologically confirmed axonal pathology, starting from 1 h postinjury and lasting up to 7 days postinjury.

In our attempt to test the hypothesis that the amount of NAA reduction is proportional to the degree of injury, we used a model of diffuse TBI producing graded neuronal and axonal damage, and we found consistent results of an acute 25% reduction of NAA in severe injury. However, the analysis of moderate injury showed nearly complete spontaneous NAA recovery after 5 days. These data are consistent with previous histological characterization of this type of injury (Foda and Marmarou, 1994), which did not reveal extensive axonal damage and was comparable to grade I diffuse axonal injury (DAI) according to Adams' classification (Adams et al., 1989). The findings are also consistent with long-term behavioral observations in moderately injured animals, which showed only slight differences compared with sham-in-

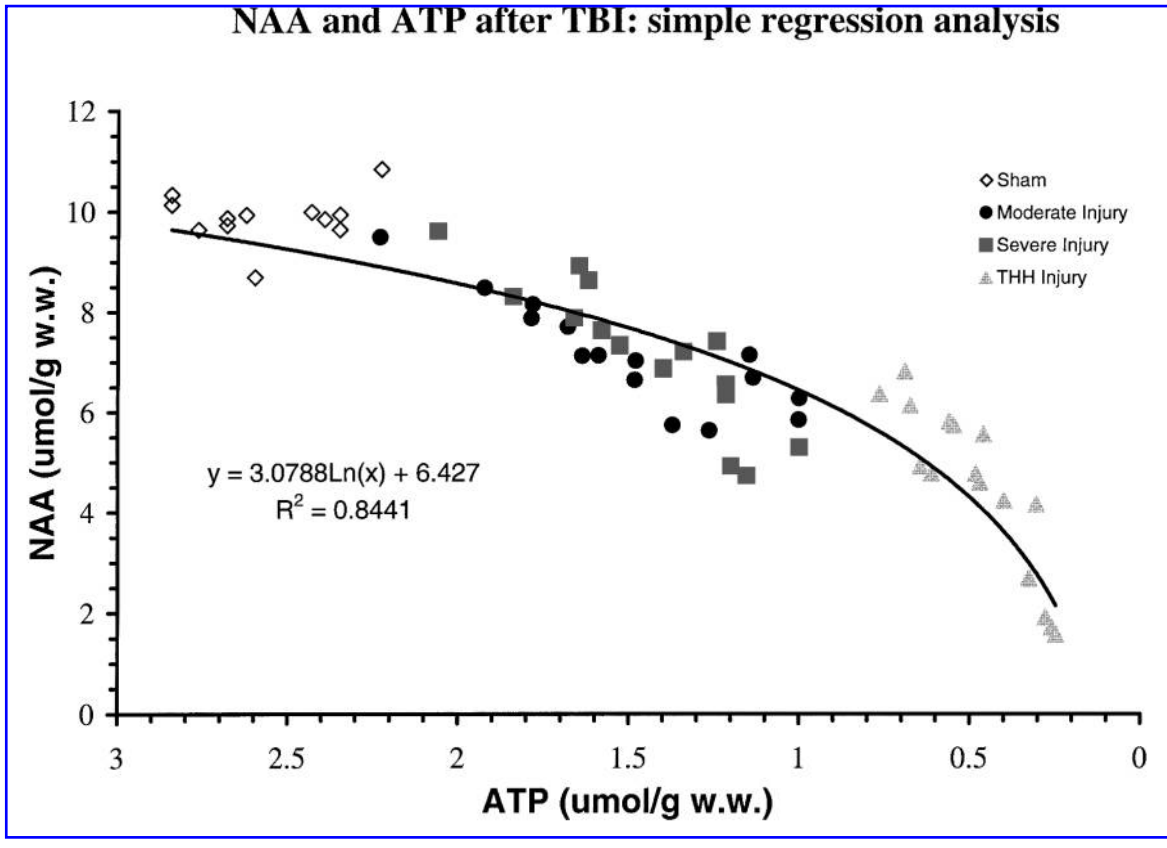

FIG. 5. Scatterplot showing the relationship between NAA and ATP variation following varying grades of diffuse traumatic brain injury. Each point represents NAA and ATP measurement in the individual animals ($n = 4$) at 2, 6, 24, and 48 h postinjury. The high correlation suggests that NAA and ATP reductions share similar mechanisms, although the nonlinear relationship reveals that these process are not tightly coupled.

jured animals, with the main differences being present 1 day postinjury with consistent improvement over time (Beaumont et al., 1999). This observation supports the potential role of NAA in quantifying neuronal damage (Cecil et al., 1998) and predicting neuropsychological outcome after TBI (Friedman et al., 1998, 1999). A previous demonstration of NAA recovery in experimentally induced DAI was reported by Rango et al. (1995) in a study that documented a 72-h reduction in NAA/Cr and a return to normal levels 7 days post injury. Finally, restoration of NAA has been observed in patients recovering from acute demyelinating lesions, stroke, epilepsy, and carotid occlusive disease in conjunction with resolution of neurological deficits (Cendes et al., 1997; De Stefano et al., 1995; Kalra et al., 1998; Uno et al., 1996).

In the present study, we measured NAA using the HPLC technique, a methodology that measures whole-brain metabolite concentrations, but which is relatively uninformative about changes at the single cell level. Hence, especially at the latest time points, it is difficult to delineate whether an absolute percentage reduction in metabolite concentration represents a uniform reduction within dysfunctional cells or rather a reduction caused by neuronal depletion with normal residual cells. Our finding of NAA recovery in the moderate injury group implies that at least one process leading to NAA reduction is reversible and not simply due to cell death. In the severely injured brain, we found almost 60% reduction in NAA; however in this model, 60% reduction in the neuronal population is not seen. A possible source of error in estimating NAA reduction at later time points is possible change in nonneuronal protein due to posttraumatic glial hypertrophy and invasion. Nevertheless, in this model of trauma, with no secondary insult, these phenomena are of limited importance in accounting for a 50% NAA reduction at 48 h (Bodjarian et al., 1997; Dietrich et al., 1999; Ito et al., 1996).

Biochemical Analysis

Absolute NAA determinations using ^{1}HMRS require either internal or external standards, each with its own advantages and limitations. A crucial assumption normally made is the use of tabulated values for brain water content, a situation clearly unsatisfactory because of differences in tissue type and the presence/absence of edema (Barker et al., 1993; Friedman, et al., 1999). This problem has led to widely varying reported levels of tissue NAA (Keevil et al., 1998) with limited work focused to document the accuracy and reliability of this technique (Florian et al., 1996; Strauss et al., 1997). In a previous report from this laboratory investigating the accuracy of an *in vivo* estimation of absolute NAA concentration by ^{1}HMRS using cerebral water as an internal reference standard, we found good agreement with the HPLC measurements only in control animals. In the traumatized animal, ^{1}HMRS NAA reduction at 4 h postinjury was less accurate with respect to HPLC (14% versus 24%, respectively; Fatouros et al., 2001).

To assess NAA concentrations, we used a new method of ion-pairing HPLC (Tavazzi et al., 2000). Under these previously described chromatographic conditions, *N*-acetylated amino acids NAA and NAG were eluted with a $k' = 6.77$ and 9.06, respectively, and were fully resolved from other compounds injected. The method was linear for concentrations of NAA and NAG ranging between 0.25 and 500 μM for both compounds (0.05–100 nmol injected). By using an appropriate buffer composition for the chromatographic elution, NAA and NAG were fully separated from other compounds commonly present in brain tissue. We can be confident that there was no cross-contamination of NAA or ATP signals, since respective absorbances were at 210- and 267-nm wavelength.

In a recent study, using the same injury model, NAA was assessed using the method described by Koller et al. (1984). In that study, values of control NAA equaled 8.490 ± 0.44 μmol/g, and surprisingly no significant variations were observed 4 h following severe TBI (8.690 ± 0.49 μmol/g w.w.; Al-Samsam et al., 2000). After adding the secondary insult, NAA concentration was only reduced by 19% (0.704 ± 0.55 μmol/g w.w), showing no difference between trauma coupled with secondary insult and secondary insult alone (HH). Those findings are in contrast to the present study, which revealed higher sham values and a proportional reduction of NAA according to the injury severity ($p < 0.0001$). Furthermore, histological analysis performed in the same model at similar time points has revealed a marked difference in neuronal damage between HH and THH (Yamamoto et al., 1999).

With the HPLC method utilized in the present paper, the lowest limit of detection for NAA was 10 pmol of injected sample. This high level of sensitivity was obtained by virtue of the diode array detector, equipped with a flow cell of 5-cm light path. We believe that the better resolution of NAA changes seen in this study relates to the greater sensitivity of this HPLC method compared with the Koller technique. It is worth recalling that the Koller technique used in the previous study does not guarantee the separation of NAA from several other brain metabolites (oxypurines, nucleosides), the concentrations of which are subjected to profound fluctuation under pathological conditions. The successful, direct, simultaneous determination of NAA and other cerebral metabo-

lites constitutes a major advantage with respect to previously described HPLC methods for NAA assay (Burri et al., 1990; Koller et al., 1984; Korf et al., 1991).

Effect of Diffuse Traumatic Brain Injury on Energetic Metabolism

One of the most important findings to arise from this study was the reversibility of NAA change following moderate brain injury and its association with restoration of ATP levels. Interestingly, NAA reduction in the early phases after trauma was comparable in all three grades of injury severity, and significant differences between the groups only fully appeared at 48 h after injury (Fig. 4). In contrast, significant differences in ATP levels were seen at earlier time points, thus suggesting that the underlying energetic disarrangement was different.

Although several studies measuring ATP using ^{31}P MRS have not been able to demonstrate a posttraumatic reduction, several reports have documented an ATP reduction following TBI, always related to mitochondrial dysfunction. Sullivan et al. (1998) reported a significant time-dependent alteration in synaptosomal mitochondria describing an immediate ATP reduction within 10 min following cortical contusion injury. Vagnozzi et al. (1999) described significant ATP, GTP, and mitochondrial nicotinic coenzyme (NAD and NADP) reduction after mild diffuse TBI starting 2 h postinjury. More recently, Ahmed et al. (2000) reported altered mitochondrial membrane potentials and a 22–28% cellular ATP reduction in mixed neuronal plus glial cultures undergoing stretch-induced injury, starting 15 min posttrauma.

A criticism of our study could arise from the observation that ATP reduction might represent a simple metabolic mismatch between energy demand and supply. However, it is important to consider that these metabolic alterations occurred in a trauma model characterized by adequate cerebral blood flow (Barzo et al., 1996; Ito et al., 1996). In this situation, a mismatch would mean a hypermetabolic state, while many studies have documented a reduced cerebral metabolic rate of oxygen ($CMRO_2$) and normal arteriovenous oxygen difference ($ADVO_2$; Bergsneider et al., 1997; Kushi et al., 1999; Martin et al., 1997; Robertson et al., 1987; Valadka et al., 2000). A marked posttraumatic increase in lactate/glucose ratio and reduced oxygen consumption (VO_2) have recently been described at 4–6 h postinjury with preserved CBF (Levasseur et al., 2000). These facts rule out an ischemic etiology but rather suggest an increase in anaerobic glycolysis in order to restore ATP, confirming that the mitochondria are dysfunctional. Finally, intracellular ATP repletion rapidly restored the resting membrane potential to normal levels, thus suggesting that mitochondrial ATP is the major limiting factor for the restoration of homeostasis after TBI (Tavalin et al., 1997). Within the monitoring time, in moderately and severely injured animals, no significant episodes of systemic hypotension or hypoxia were reported, thus excluding a possible ischemic etiology. Although from our data we cannot exclude local ischemic phenomena, possibly mediated by various molecules (cathecolamines, entdothelin, cytokines) rather than due to the lack of substrates, ATP deficiency seems related to the extent of mitochondrial damage directly triggered by the traumatic insult (Fiskum, 2000; Vagnozzi et al., 1999; Xiong et al., 1997). As a clinical implication, it is noteworthy to recall that, in the majority of TBI patients (65%), neurological function is transiently or permanently lost in the presence of adequate cerebral blood flow (Obrist et al., 1984).

N-Acetylaspartate as a Potential Marker of Mitochondrial Dysfunction

Previous studies have furnished strong evidence to support the view that NAA reduction is not only related to neuronal death, but also could serve as an indicator of mitochondrial dysfunction (Brenner et al., 1993; Clark, 1998; Goldstein, 1969; Heales et al., 1995; Knizley, 1967; Patel and Clark, 1979; Saragea et al., 1965). Recently, a close linear relationship between ATP synthesis and the ability to synthesize NAA was described (Bates et al., 1996).

In our study, the high statistical correlation between ATP and NAA levels ($R^2 = 0.84$) would suggest that depletion of NAA and ATP following TBI share a common process. However, the metabolites' relationship was not linear (Fig. 5), which also suggests that the two compounds are not tightly coupled. The fact that NAA and ATP recovery was seen only following moderate trauma indicates that there may be a threshold of ATP depletion, beyond which NAA recovery is prevented.

In a recent report (Braun et al., 1999) in which both ^{1}H and ^{31}P MRS were performed in a model of experimentally induced chronic hydrocephalus, NAA reduction was not accompanied by a concomitant ATP reduction, although a significant increase in lactate was described. We believe that these data are not in conflict with our results and only support the notion that spectroscopic ratio data are still approximate and difficult to interpret. The levels of NAA reduction in Braun's study are comparable with our moderate injury data at 5 days, in which NAA was reduced by 14% and ATP was normal. We agree with Braun's interpretation that moderate NAA

reductions may not reveal significant ATP depletion since ATP synthesis may be compensated for by an increase in anaerobic metabolism.

Our findings of NAA reduction within 1-min posttrauma are interesting. There is substantial evidence in the literature supporting the rapidity of the effect of a mechanical insult on mitochondrial dysfunction (Sullivan et al., 1998), which may be largely a calcium-dependent process occurring immediately after trauma (LaPlaca and Thibault, 1998; Peng and Greenamyre, 1998). However, the present study has a limitation due to the fact that mitochondrial function was not directly measured. Although NAA synthesis is energy-dependent and requires ATP availability (Baslow, 1997), further studies are necessary to establish a causal relationship between the two metabolites and to exclude the possibility of examining two epiphenomenon of the same process. The highly similar time course would suggest that measurement of NAA as an informative surrogate marker of tissue energetic metabolic dysfunction is useful. However, further studies are necessary to investigate the relationship between NAA reduction and posttraumatic mitochondrial dysfunction.

CONCLUSION

The reduction of NAA following diffuse TBI is proportional to the severity of traumatic insult. NAA falls within minutes of injury and potentially may recover if the traumatic insult is moderate. Adjunctive hypoxic-hypotensive insult strongly exacerbates the biochemical damage blunting the recovery process. The concomitant reduction of NAA with ATP observed in this model of diffuse injury provides supportive evidence that NAA is decreased as result of energetic disturbances. Most importantly, recovery of NAA has been observed only in concert with restoration of ATP. These data imply that NAA reduction can be a reversible phenomenon, and further studies are necessary to demonstrate whether pharmacological attempts to preserve mitochondrial function will affect NAA recovery and ultimately the prognosis of diffuse TBI.

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