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SUPPRESSION OF THE INDUCIBLE FORM OF NITRIC OXIDE SYNTHASE PRIOR TO TRAUMATIC BRAIN INJURY IMPROVES CYTOCHROME *c* OXIDASE ACTIVITY AND NORMALIZES CELLULAR ENERGY LEVELS

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Abstract—We have previously shown that the observed immediate increase in nitric oxide (NO) plays a significant role in the control of the cerebral microcirculation following traumatic brain injury (TBI). However, a second consequence of increased NO production after TBI may be impaired mitochondrial function, due to the fact that NO is a well-known inhibitor of cytochrome *c* oxidase (CcO). CcO is a key enzyme of the mitochondrial oxidative phosphorylation (OxPhos) machinery, which creates cellular energy in the form of ATP. NO competes with oxygen at the heme a_3–Cu_B reaction center of CcO. We thus hypothesized that TBI triggers inhibition of CcO, which would in turn lead to a decreased energy production by OxPhos at a time of an elevated energy demand for tissue remodeling. Here we show that TBI as induced by an acceleration weight drop model of diffuse brain injury in rats leads to CcO inhibition and dramatically decreased ATP levels in brain cortex. CcO inhibition can be partially restored by application of iNOS antisense oligonucleotides prior to TBI, which leads to a normalization of ATP levels similar to the controls. We propose that a lack of energy after TBI caused by inhibition of CcO is an important aspect of trauma pathology.

Key words: trauma, mitochondria, iNOS, cytochrome *c* oxidase, nitric oxide, ATP.

The effect of brain trauma on mitochondrial function is not well understood. However, two aspects of traumatic injury suggest a role of this organelle in trauma pathology. First, ischemia and hypoperfusion following trauma (Nortje and Menon, 2004) lead to oxygen deprivation. Second, increased nitric oxide (NO) production is, at least in part, mediated through the up-regulation of the inducible form of nitric oxide synthase (iNOS) in the sensorimotor cortex (SmCx), as we have previously shown (Petrov et al., 2000). Both a reduction in oxygen levels and an increase in NO levels would affect cytochrome *c* oxidase (CcO), a key enzyme of aerobic energy production in the mitochondria. This is so because oxygen is the substrate of CcO whereas NO is an inhibitor of CcO.

The impact of NO on mitochondrial function has been well established over the last two decades (Brown, 2001; Brookes, 2004; Gorren and Mayer, 2006), and CcO appears to be a key target. CcO is the terminal enzyme of the respiratory chain, accepting electrons from cytochrome *c* and transferring them to molecular oxygen, which is reduced to water. This reaction is coupled to the pumping of protons across the inner mitochondrial membrane. Together with NADH dehydrogenase and bc_1 complex, CcO generates the mitochondrial membrane potential $\Delta\Psi_m$, which is utilized for the synthesis of ATP through ATP synthase. The entire process is called oxidative phosphorylation (OxPhos), which provides more than 15 times more cellular energy in the form of ATP compared with the glycolytic pathway. The mammalian brain fully depends on aerobic energy production and any impact disturbing this process, such as oxygen withdrawal or stroke, can have devastating consequences.

Mammalian CcO has been crystallized as a dimer, which contains 13 subunits per monomer (Tsukihara et al., 1996). The mammalian enzyme consists of 10 nuclear-encoded subunits in addition to the three largest mitochondrial-encoded subunits, which contain the four catalytic redox centers. NO reversibly inhibits CcO through competition with oxygen at the binuclear heme a_3–Cu_B oxygen binding site (Brown and Cooper, 1994; Giulivi, 1998). In addition to trauma (Steiner et al., 2004), many human diseases are associated with increased cellular NO levels, including arthritis (Cuzzocrea, 2006), sepsis and septic shock (Assreuy, 2006), cancer (Fukumura et al., 2006), obesity, and diabetes (Stepp, 2006). Despite other actions of NO in the cell, its inhibitory action on CcO might, at least in part, explain some aspects of NO-related pathological conditions, because inhibition of oxidative energy production would decrease cellular energy levels.

We hypothesized that iNOS-triggered NO production after TBI affects mitochondrial function. We show that CcO is inhibited after TBI and that ATP levels are significantly

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Abbreviations: ANOVA, analysis of variance; CcO, cytochrome *c* oxidase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; ETC, electron transport chain; iNOS, inducible form of nitric oxide synthase; NO, nitric oxide; ODN, oligodeoxynucleotide; OxPhos, oxidative phosphorylation; PVDF, polyvinylidene fluoride; SmCx, sensorimotor cortex; TBI, traumatic brain injury; TN, turnover number.

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reduced. Suppression of iNOS expression partially restores CcO activity and fully restores ATP levels. We propose that energy paucity during trauma may be an important aspect of disease pathology since tissue remodeling and normalization of cell function after traumatic impact are energy intensive.

EXPERIMENTAL PROCEDURES

Infusion of antisense oligodeoxynucleotides (ODNs)

In this study we used male Sprague–Dawley rats (250–300 g). Ten to twelve hours before injury animals were infused intracerebroventricularly, using a 26-guage 10 μL Hamilton syringe (Hamilton Company, Reno NV, USA) (coordinates from bregma based on Paxinos and Watson, 1998 = −0.4 mm anterior/posterior, ±1.5 mm medial/lateral, −3.4 mm ventral) once with 2 nmol (in 2 μL) of phosphorothioated antisense rat iNOS ODNs (GGC AAG CCA TGT CTG) or with randomly sequenced, control ODNs (cODNs with the following sequence CGT CCC TAT ACG ACC). Both ODNs were obtained from Biognostik, Göttingen, Germany. The control sequence is not complimentary to any other known mRNA. The ODNs were labeled with fluorescein isothiocyanate (FITC) for detection in individual cells. In general, the infusions were made as previously described (Petrov et al., 1995). Briefly, animals were anesthetized with a mixture of ketamine and xylazine (0.8 mL/kg and 0.25 mL/kg, respectively), placed in a stereotaxic frame, and a small hole was drilled over the right hemisphere. A guiding cannula was placed in the lateral ventricle (1.6 mm posterior to the bregma, 2.4 mm lateral to the midline, and 5.1 mm ventral from the surface of the skull) to reach the lateral ventricle according to the stereotaxic atlas of Paxinos and Watson (1998). ODNs were delivered through an internal cannula attached to a Hamilton syringe. The internal cannula was left in place for 1 min following delivery. The hole in the skull was filled with dental cement and the skin sutured. All experiments conformed to Wayne State University Animal Investigation Committiee guidelines on the ethical treatment of animals, and to the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. The numbers of animals used were as small as possible, consistent with statistical significance. Any suffering the animals might undergo was alleviated as much as possible.

Induction of traumatic brain injury (TBI)

TBI was induced by dropping a weight (450 g) from 2 m onto a steel helmet attached to the skull of the animal (a modified Marmarou's model, Marmarou et al., 1994). Prior to TBI animals were anesthetized with 5% halothane by intratracheal intubation as described previously (Petrov et al., 2000). Animals were maintained under anesthesia with a mixture of halothane (1%) and N_2O/O_2 in a ratio of 2:1. Body temperature was maintained at 37 °C with a heating pad and measured with a rectal thermometer. After injury animals were kept under 1.5% halothane anesthesia until spontaneous respiration resumed. Recovery was assessed by postinjury behavioral injury assessments (PINA) as described previously (Petrov et al., 2000). Control animals were treated identically, except that TBI was not inflicted.

iNOS *in situ* hybridization

Twelve sections per animal were randomly selected from serial 12 μm cryostat-cut sections through the SmCx (−0.26 mm to −2.23 mm from bregma based on coordinates from Paxinos and Watson, 1998), dehydrated in graded ethanol, and incubated with a HybriBuffer ISH prehybridization solution (4× SSC, different fractions of sheared DNA and stabilizing RNA from Chemicon Inc., Temecula, CA, USA) at 30 °C for 2 h as previously described (Petrov et al., 2000). HybriBuffer ISH containing 10^6 cpm ^{35}S-radiolabeled antisense iNOS oligonucleotides (Chemicon Inc., cat. # H-215.541) per section was used for hybridization. Alternate sections were hybridized with sense or mismatch mRNA (Chemicon Inc.). No specific labeling was observed in these control tissue sections. Hybridization was carried out in a moist chamber at 30 °C for 16–18 h. Subsequently tissues were washed three times in a solution containing 1× SSC (5–6 min at room temperature) followed by a rinse (twice for 15 min at 39 °C) in a solution of 0.1× SSC containing 0.05% beta-mercaptoethanol. The signal was detected autoradiographically by dipping the slides in Kodak NTB-2 emulsion (1:1 in dH_2O at 42 °C) and after 5 days they were developed in D19 and fixed in Kodak Rapid fixer. Semiquantitative analysis was achieved using optical densitometry as described previously (Petrov et al., 2000).

Western blot analysis

To determine iNOS protein content, tissue blocks containing the SmCx were collected from three animals per group and treated as previously described (Rafols et al., 2004). Briefly, tissue blocks were homogenized and the protein concentration was determined from the extracts after centrifugation using the Bradford assay. Fifty micrograms of protein was subjected to a denaturing polyacrylamide gel electrophoresis. Protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane (Osmotics, Westborough, MA, USA). The PVDF membranes were incubated with anti-iNOS antiserum (1:500, Santa Cruz Biotechnol., Santa Cruz, CA, USA) followed by incubation with peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA), and signals were detected with ECL system (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). A complete abolition of the immunoreactivity was observed following pre-incubation of the primary antibody with a specific blocking peptide.

CcO histochemistry

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. CcO activity was measured on rat brain sections derived from SmCx (12 μm) derived from fresh frozen rat brains 4 h, 24 h, and 48 h after TBI and controls. We applied an established protocol with a few modifications (Wong-Riley, 1979). Briefly, slides were transferred from −80 °C to room temperature and tissue sections were immediately circled with a hydrophobic pen, allowing the application of small reaction buffer volumes of 100 μL per section or less. Slides were transferred to a moist chamber and 100 μL of freshly prepared reaction buffer (100 mM KH_2PO_4, pH 7.4, 4% sucrose, 0.50 mg/mL diaminobenzidine (DAB), 200 μg/mL catalase, 0.15 μg/mL cow heart cytochrome *c*) was added on each section and incubated for 20 min at 37 °C in the dark. The reaction was stopped by washing the slides in 100 mM KH_2PO_4 (pH 7.4) three times. Slides were rinsed once in dH_2O and air dried for 3 h. Slides were mounted with Paramount. For semiquantitative assessment of the intensity of the CcO histochemical reaction images were captured using a Leica Axiophot light microscope. Images (20–30 through the cortex of each animal) were analyzed with the MetaMorph (Fryer, Huntley, IL, USA) image analysis system and the relative intensity of the signal was determined. An absolute value was obtained by subtracting the background intensity (measured for each individual section) from the intensity registered in the areas of interest. Intensities per area (three areas per section were analyzed) were averaged for each animal. Data from each animal were then averaged per experimental group (S.E.M.=differences between animals per group). Statistical analysis (analysis of variance (ANOVA)) was used to determine differences between groups. Significant difference

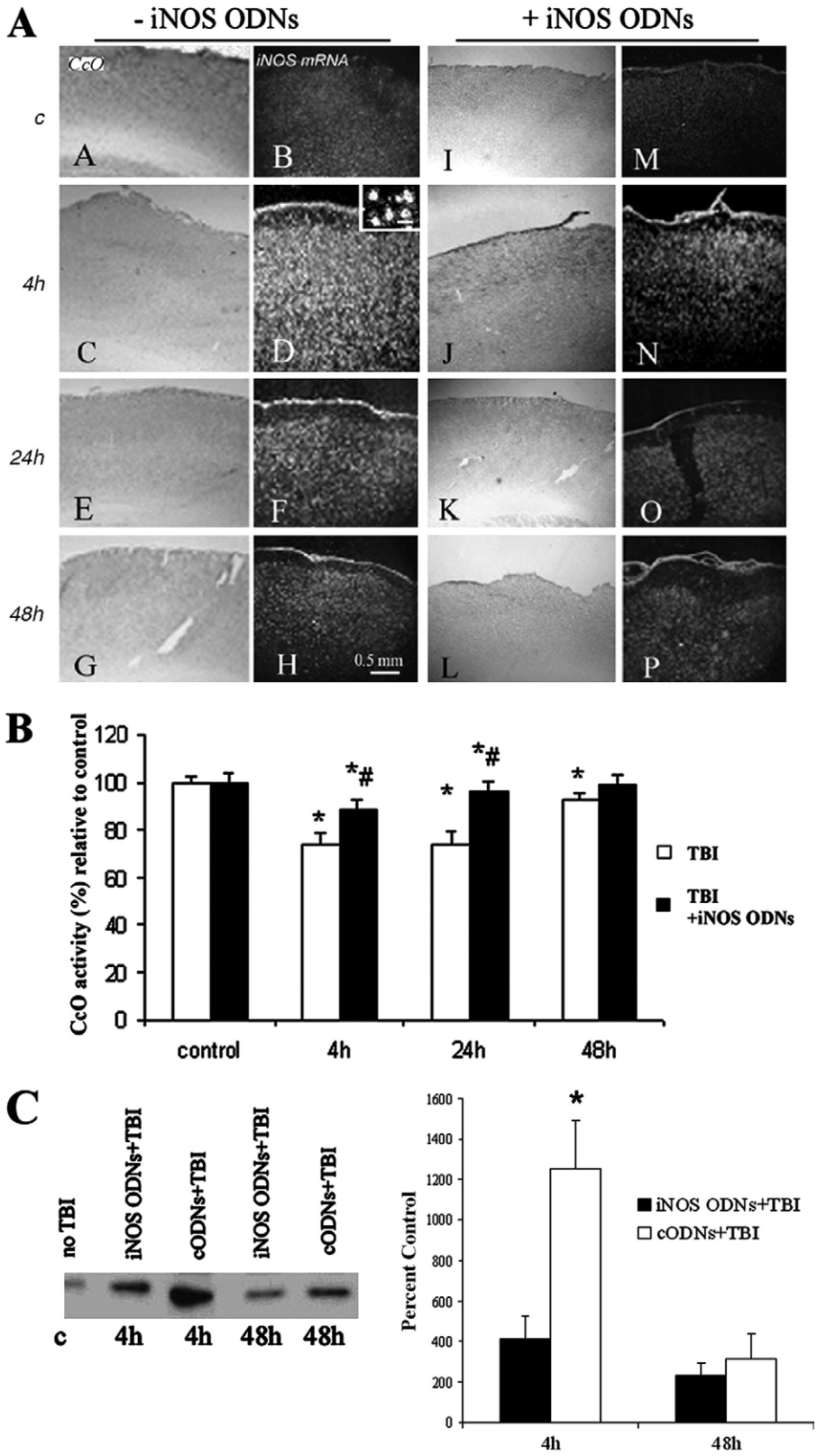

Fig. 1. CcO activity is inhibited after TBI, and can be partially restored by iNOS suppression. (A) Photomicrographs depicting histochemical CcO activity (A–G and I–L, brightfield) and iNOS mRNA labeling (B–H and M–P, darkfield) on adjacent sections in animals that have been subjected to TBI only (C–H) and animals that have been pretreated with iNOS antisense ODNs prior to TBI (J–P). Inhibition of iNOS results in greater intensity of the CcO activity in J–L, which was reduced by ~30% in the animals that were not pretreated with ODNs (C, E, G). Note that in the control animals

($P<0.05$) between groups was established by the use of the Student-Neuman-Keuls post hoc analysis.

CcO activity measurements using rat brain tissue

CcO activity was determined as previously described (Lee et al., 2005) with slight modifications. Frozen brain SmCx (50 mg) from rats was solubilized in 500 μL chilled measuring buffer (10 mM K–Hepes (pH 7.4), 40 mM KCl, 1% Tween 20, 2 μM oligomycin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM KF, 1 mM Na-vanadate, 2 mM EGTA) using a Teflon microtube pestle applying five strokes followed by sonication (microtip, 3×10 s pulses). Cell debris was removed by centrifugation (2 min, 16,000×*g*) and the supernatant was used for respiration measurements. Protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA). CcO activity was determined at 25 °C in a closed 200 μL chamber containing a micro Clark-type oxygen electrode (Oxygraph system, Hansatech, Pentney, King's Lynn, Norfolk, UK). Since CcO is regulated by adenine nucleotides (Napiwotzki et al., 1997) all measurements were performed under controlled conditions in the presence of 5 mM ADP or 5 mM ATP including an ATP regenerating system as described (Lee et al., 2002). Oxygen consumption was monitored on a computer and calculated using the Oxygraph software (Hansatech). Turnover number (TN) is defined as consumed O_2 (μM)/(min·total protein (mg)).

Determination of cellular ATP levels

For ATP measurements, SmCx samples from controls and from rats after TBI in the presence or absence of iNOS ODNs were immediately excised after killing the animals, instantly frozen in liquid nitrogen, and stored at −80 °C until measurement. In order to release cellular ATP, frozen tissue (40 mg) was boiled for 2 min after the addition of 300 μL boiling buffer (100 mM Tris–Cl (pH 7.75), 4 mM EDTA). Samples were put on ice and homogenized by sonication (microtip, 1×10 s pulse). ATP concentrations were determined in triplicate per animal using the ATP bioluminescence assay kit HS II (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Data were standardized to the protein concentration, which was determined as described above. Data were expressed as the mean±standard error of the mean (S.E.M.) over three independent measurements per animal. Significant differences between multiple groups were determined using one-way ANOVA followed by least significant difference (LSD) post hoc analysis. All analyses were performed using SPSS version 8.0.

RESULTS

TBI leads to a strong up-regulation (3215%±127% of control as determined by optical densitometry) of the iNOS mRNA in a time-dependent manner (Fig. 1A, second column), followed by increased iNOS protein levels (Fig. 1C). iNOS transcript levels are maximal at 4 h post-TBI, then decrease over time but are still well above control levels 48 h post-TBI. Since NO is a competitive inhibitor of mitochondrial CcO we hypothesized that CcO is inhibited post-TBI. We determined CcO activity on serial sections using the histochemical method at 4 h, 24 h, and 48 h after TBI (Fig. 1A, first column). At 4 h and 24 h, CcO activity was reduced to 74% compared with the controls, and improved to 93% of control activity 48 h after TBI (Fig. 1B).

To test whether or not CcO inhibition was caused by up-regulation of iNOS we determined CcO activity in serial brain sections from animals that were injected with iNOS antisense oligonucleotides prior to TBI. Fig. 1A (fourth column) shows that iNOS transcription was partially suppressed after antisense oligonucleotide application and TBI compared with TBI alone, and a similar result was obtained for the iNOS protein (Fig. 1C, lanes 2 and 4). At the same time CcO activity is partially restored from 74% to 89% at 4 h and further improves to near normal levels, to 96% and 99% of control activity, at 24 and 48 h post-TBI (Fig. 1A, compare I with J–L, and Fig. 1B).

In addition to direct inhibition of CcO by NO, other mechanisms to reduce CcO activity are possible, such as a down-regulation of the CcO protein. Although mitochondrial turnover is a slow process, we tested for differences in aerobic capacity of cortex tissue of control and 4 h post-TBI animals, because iNOS induction and CcO inhibition were maximal at that time point. Fig. 2 shows measurements of CcO activity using microrespirometry via the polarographic method. This approach involves breaking up the tissue followed by washing steps, and thus the removal (i.e., a strong dilution) of NO from the sample directly before measurement (see Experimental Procedures). We measured CcO activity in the presence of allosteric CcO activator ADP and inhibitor ATP (Fig. 2, closed and open symbols, respectively): ATP respiration was about 50% inhibited in comparison to ADP respiration at maximal turnover. Importantly, for both inhibited and activated respiration there was no detectable difference between animals subjected to TBI and the controls. In addition the presence of iNOS antisense oligonucleotides did not have an effect on CcO activity. Taken together the results presented in Figs. 1 and 2 suggest that aerobic capacity did not change during the first 4 h post-TBI, and that CcO inhibition as observed after TBI is a direct effect of NO on CcO.

CcO is the terminal enzyme of the mitochondrial electron transport chain (ETC) and it was proposed to be the rate-limiting enzyme of the overall process under physiological conditions (reviewed in Kadenbach et al., 2004). We thus hypothesized that inhibition of this central enzyme should result in an overall inhibition of ETC activity. Since the ETC generates the mitochondrial membrane potential, which is used by ATP synthase to produce ATP, we determined cellular ATP levels pre- and post-TBI. Because ATP turnover is very fast, special care was taken for these

(B) and in animals that have only received iNOS ODNs there is no expression of iNOS mRNA. At higher power individual cells can be detected (inset in D, scale bar=25 μm). Negative control for CcO activity that included CcO inhibitor KCN (1 mM) did not show any staining (not shown). (B) Quantitative analysis of slides stained for CcO activity ($n=4$ for each condition). * $P<0.05$ compared with control; # $P<0.05$ compared with TBI. (C) Western analysis and semiquantification of $N=3$ animals with an iNOS antibody shows strongly induced iNOS protein levels 4 h post-TBI (lane 3) (1257% of control ±234% as determined by semiquantitative optical densitometry), which decreased over time (48 h, lane 5). Application of iNOS ODNs can partially suppress iNOS expression (lanes 2 and 4), whereas control ODNs (cODNs) do not show this effect (lanes 3 and 5).

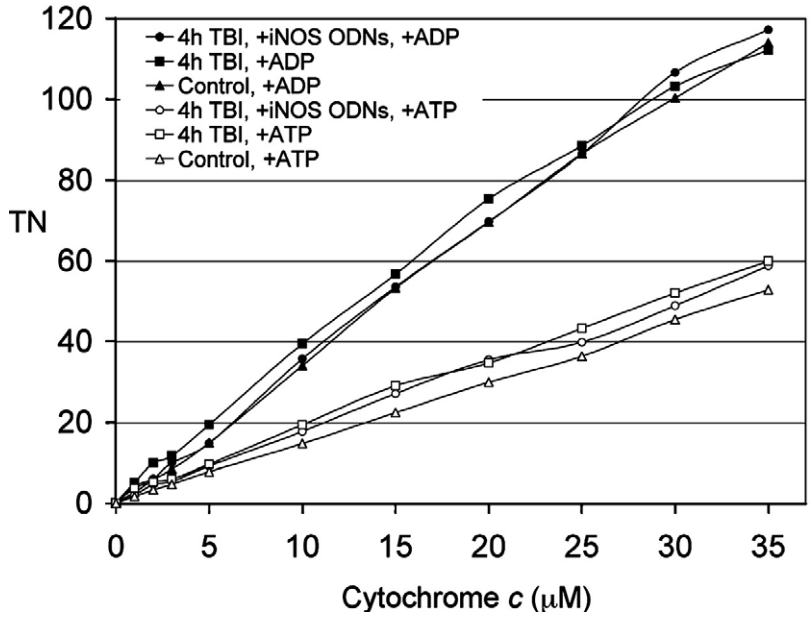

Fig. 2. Aerobic capacity of brain tissue is unchanged 4 h post-TBI. SmCx from control rats (triangles) and animals after TBI in the absence or presence of iNOS antisense ODNs (squares and circles, respectively) was homogenized and CcO activity was measured by the addition of increasing amounts of cytochrome *c*. Reaction was performed in the presence of allosteric CcO activator ADP (filled symbols) or allosteric inhibitor ATP (open symbols). TN is defined as consumed O_2 (μM)/(min·total protein (mg)). No differences were observed between samples, suggesting that the amount of CcO has not changed after TBI. Shown are six representative measurements (n=3).

measurements and brain cortex was immediately flash-frozen after the animals were killed and stored at −80 °C until measurement. Importantly, ATP levels were dramatically reduced to 38% compared with the control at 4 h post-TBI, but levels were indistinguishable from the controls when animals were pretreated with iNOS antisense oligonucleotides (Fig. 3). A similar pattern was obtained at 24 h post-TBI. At 48 h post-TBI ATP levels were still significantly reduced to 65% in comparison to the controls but clearly improved versus the two earlier time points. For all time points post-TBI, the suppression of iNOS through the application of antisense oligonucleotides was associated with a normalization of cellular energy levels.

DISCUSSION

Very little is known about the effect of TBI on mitochondrial function. An earlier study found impairment of the mitochondrial OxPhos process in brain trauma patients whereas a patient with intracranial hypertension and a patient with epilepsy did not show such impairment (Verweij et al., 1997).

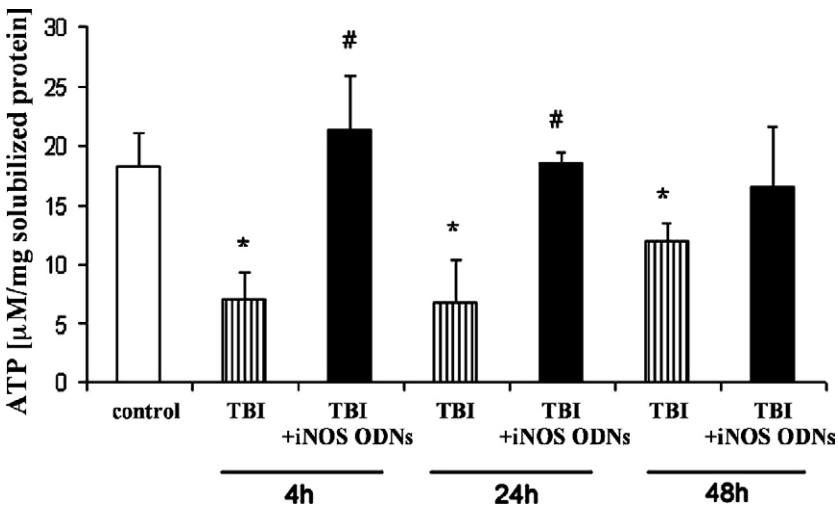

Fig. 3. Rat SmCx shows strongly decreased cellular ATP levels after TBI, which can be restored by suppression of iNOS expression. ATP concentrations were determined with the bioluminescence method using rat SmCx tissue from animals 4, 24, and 48 h post TBI (pretreated with or without iNOS antisense ODNs) and controls. * $P<0.05$ compared with control; # $P<0.05$ compared with TBI.

The authors suggested that a cytosolic calcium overload was leading to an excessive calcium uptake by the mitochondria causing detrimental effects inside the organelle. However, calcium is considered a strong activator of mitochondrial function and OxPhos (Kadenbach et al., 2004), most likely mediated by calcium-activated protein phosphatases leading to dephosphorylation and activation of mitochondrial proteins (Hopper et al., 2006). A possible explanation of the observed inhibitory effect of increased calcium on OxPhos after trauma may be through an amplification of NO production: there is calcium-dependent and -independent NO production by NOS enzymes (Louin et al., 2004), and lowering the calcium concentration would decrease NO concentrations, which in turn would relieve the inhibitory effect on CcO and allow increased energy production by OxPhos. This model would also explain observed beneficial effects of selective N-type calcium channel blocker SNX-111 on mitochondrial function (Verweij et al., 1997).

The important role of NO leading to mitochondrial dysfunction is further supported by work presented here. This report confirms our previous findings that in an acceleration impact model of TBI, iNOS mRNA was detected in neurons as well as cortical vascularization (Petrov et al., 2000; Petrov and Rafols, 2001). Interestingly, this contrasts with data from stroke and other TBI models which show that iNOS is primarily found in the vascularization, infiltrating inflammatory cells, and in other non-neuronal cell types (Clark et al., 1996; Iadecola et al., 1996). The lack of iNOS mRNA detected in inflammatory cells may be explained by the fact that no reports have shown a significant inflammatory response following impact using Marmarou's model of TBI (Rafols et al., 2007). The presence of iNOS mRNA within neurons, as previously suggested (Petrov et al., 2000), may facilitate maximal expression of vascular iNOS via axonal delivery of iNOS to the neighboring vascular wall, leading to overproduction of NO and subsequent mitochondrial dysfunction in our model of TBI.

Although it is well-accepted that brain ischemia and hypoperfusion are key aspects of trauma-related pathology (Nortje and Menon, 2004), mitochondrial CcO, the enzyme that utilizes more than 90% of cellular oxygen, has not been analyzed in a trauma context. In addition, upregulation of iNOS post-TBI, followed by increased NO production, suggests an effect on CcO due to the known capabilities of NO as an inhibitor competing with oxygen at the reaction site in the catalytic subunit I of CcO. Even 48 h post-TBI ATP levels only partially improved and were still 35% reduced compared with control animals, which may contribute to the previously reported cellular damage within the SmCx (Rafols et al., 2007). CcO activity, which was measured on brain tissue sections, was significantly inhibited by 26% 4 h post-TBI. This effect was not caused by changes in the aerobic capacity of the brain tissue, suggesting that CcO protein levels do not change post-TBI. Most likely, CcO inhibition *in vivo* is even more pronounced because our *in vitro* CcO activity measurement does not account for oxygen deprivation as a result of brain ischemia and hypoperfusion: the CcO histochemistry assay utilizes oxygen-saturated reaction solutions, which are overlaid on the tissue sections, and due to the large surface to volume ratio oxygen cannot reach realistically low levels as may be observed after TBI in the hypoperfused cortex of a live animal.

Under normal conditions there is some excess oxygen capacity in brain mitochondria, but even short and transient periods of anoxia lead to changes in the oxidation state in CcO as has been demonstrated with near-infrared and ^{31}P NMR spectroscopy in piglets (Springett et al., 2003). The enormous energy demand of the brain explains its exquisite reliance on aerobic energy production. It is, therefore, not surprising that changes in oxygen availability in combination with increased production of CcO inhibitor NO have a strong impact on aerobic energy production, as we show in an animal model for TBI.

Interestingly, application of iNOS antisense oligonucleotides, which led to a suppression of iNOS mRNA production, significantly improved CcO activity and fully restored ATP levels. Future work will determine whether iNOS antisense oligonucleotides can, ultimately, reduce the extent of cellular damage seen in the SmCx. If, as we speculate, decreased cellular energy levels exacerbate cell performance and impede energetically costly tissue remodeling, suppression of iNOS at the transcript or protein level might be an attractive target for future trauma therapy.

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