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N-acetylcysteine amide preserves mitochondrial bioenergetics and improves functional recovery following spinal trauma

Samir P. Patel [a,1], Patrick G. Sullivan [b,1], Jignesh D. Pandya [b], Glenn A. Goldstein [c], Jenna L. VanRooyen [a], Heather M. Yonutas [b], Khalid C. Eldahan [a], Johnny Morehouse [d], David S.K. Magnuson [d], Alexander G. Rabchevsky [a,*]

[a] Spinal Cord and Brain Injury Research Center, Department of Physiology, University of Kentucky, Lexington, KY 40536-0509, USA
[b] Spinal Cord and Brain Injury Research Center, Department of Anatomy & Neurobiology, University of Kentucky, Lexington, KY 40536-0509, USA
[c] Pediatric Endocrinology, Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden
[d] Departments of Neurological Surgery, Anatomical Science, and Neurobiology, University of Louisville, Louisville, KY 40292, USA

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ABSTRACT

Mitochondrial dysfunction is becoming a pivotal target for neuroprotective strategies following contusion spinal cord injury (SCI) and the pharmacological compounds that maintain mitochondrial function confer neuroprotection and improve long-term hindlimb function after injury. In the current study we evaluated the efficacy of cell-permeating thiol, N-acetylcysteine amide (NACA), a precursor of endogenous antioxidant glutathione (GSH), on mitochondrial function acutely, and long-term tissue sparing and hindlimb locomotor recovery following upper lumbar contusion SCI. Some designated injured adult female Sprague–Dawley rats (n = 120) received either vehicle or NACA (75, 150, 300 or 600 mg/kg) at 15 min and 6 h post-injury. After 24 h the total, synaptic, and non-synaptic mitochondrial populations were isolated from a single 1.5 cm spinal cord segment (centered at injury site) and assessed for mitochondrial bioenergetics. Results showed compromised total mitochondrial bioenergetics following acute SCI that was significantly improved with NACA treatment in a dose-dependent manner, with maximum effects at 300 mg/kg (n = 4/group). For synaptic and non-synaptic mitochondria, only 300 mg/kg NACA dosage showed efficacy. Similar dosage (300 mg/kg) also maintained mitochondrial GSH near normal levels. Other designated injured rats (n = 21) received continuous NACA (150 or 300 mg/kg/day) treatment starting at 15 min post-injury for one week to assess long-term functional recovery over 6 weeks post-injury. Locomotor testing and novel gait analyses showed significantly improved hindlimb function with NACA that were associated with increased tissue sparing at the injury site. Overall, NACA treatment significantly maintained acute mitochondrial bioenergetics and normalized GSH levels following SCI, and prolonged delivery resulted in significant tissue sparing and improved recovery of hindlimb function.

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Introduction

Traumatic spinal cord injury (SCI) induces a cascade of secondary pathophysiological events that results in neuronal death at and around the site of injury, with accompanying loss of motor and sensory functions. Mitochondrial dysfunction is thought to be one of the major cause–effect factors for these secondary events (Benedict et al., 2012; Dumont et al., 2001; Huang et al., 2012; Patel et al., 2009, 2010, 2012; Sullivan et al., 2007; Teng et al., 2004), and thus can serve as a pivotal target for pharmacological strategies to foster neuroprotection after SCI. Mitochondria are known as the "powerhouse" of the cell which generates cellular energy in the form of ATP. On the other hand, they are also a major site for free radical production as well as a primary target for free radical attack, and we have documented the progressive nature of mitochondrial dysfunction and their oxidative damage over 24 h post-SCI (Sullivan et al., 2007). Importantly, we have shown that administration of pharmacological compounds that maintain mitochondrial function confers neuroprotection and significantly improves long-term hindlimb function after injury (Patel et al., 2010, 2012).

In the current study we assessed the neuroprotective efficacy of a novel thiol-containing antioxidant, N-acetylcysteine amide (NACA). NACA is a modified form of its parent compound N-acetylcysteine (NAC) that is a precursor of the most abundant endogenous antioxidant, glutathione (GSH) (De Flora et al., 1991). Depletion of GSH has been associated with conditions such as aging, diabetes mellitus, inflammation, and neurodegenerative diseases as well as CNS injuries (Drake et al.,

* Corresponding author at: Spinal Cord & Brain Injury Research Center (SCoBIRC) B471, Biomedical & Biological Sciences Research Building, 741 South Limestone Street, Lexington, KY 40536-0509, USA. Fax: +1 859 257 5737.
E-mail address: agrab@uky.edu (A.G. Rabchevsky).
[1] The first two authors contributed equally to this study.

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2002; Droge, 2002; Kamencic et al., 2001; Thomas and Mallis, 2001). Thus, thiol-containing compounds have received growing attention due to the profound role of intracellular GSH in cellular antioxidant defense systems. Accordingly, NAC supplementation has been shown to have beneficial effects on oxidative stress induced diseases and clinical disorders (Arent et al., 2012; Foltz et al., 2012; Holdiness, 1991; Kelly, 1998; Parcell, 2002). Experimental evidence suggests that NAC facilitates biosynthesis of intracellular GSH by reducing extracellular cystine to cysteine (Issels et al., 1988). NAC, itself, can serve as a potent free radical scavenger due to its nucleophilic interactions with reactive oxygen species (ROS) (Aruoma et al., 1989; Ozaras et al., 2003). However, the carboxyl group of NAC loses its proton at physiological pH, which makes the compound negatively charged and reduces its permeability through biological membranes. Thus, bioavailability of NAC is very low, efforts were made to synthesize a modified compound NACA (an amide form of NAC) that possesses a neutral charge at physiological pH (Grinberg et al., 2005; Offen et al., 2004). NACA is more membrane permeable than NAC and reacts with the oxidized form of glutathione (GSSG) to generate GSH, possibly by exchanging disulfide with GSSG (Grinberg et al., 2005). NACA has been shown to cross the blood–brain barrier, chelate copper, scavenge free radicals and attenuate experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein inoculation (Offen et al., 2004). Moreover, NACA protects mammalian cells in vitro from oxidants such as HIV proteins, glutamate and beta amyloid toxicity (Bartov et al., 2006; Penugonda et al., 2005; Price et al., 2006).

Based on these antioxidant properties of NACA and our reports that maintenance of mitochondrial function following SCI is neuroprotective (Patel et al., 2010, 2012), we herein investigated the protective effects of NACA on the mitochondrial GSH pool, acute mitochondrial function, long-term tissue sparing, and hindlimb locomotor function following upper lumbar (L1/L2) contusion SCI in adult rats. Importantly, we employed refined gait analyses to assess the functional recovery in addition to a conventional locomotor rating scale. We also report a novel method for the isolation of synaptic (neuronal), non-synaptic (neuronal somata and glia) and mixed population of synaptic + non-synaptic mitochondria from a single 1.5 cm of thoracolumbar spinal cord segment to assess the effect of NACA treatment on their bioenergetics using the Seahorse Bioscience XF24 Extracellular Flux Analyzer and measuring activities of mitochondrial enzyme complexes: NADH dehydrogenase (Complex I), cytochrome c oxidase (Complex IV) and pyruvate dehydrogenase (PDHC).

Materials and methods

Spinal cord injury and treatments

Female Sprague–Dawley rats (n = 141, see Table 1 for detail) (Harlan Labs, IN) weighing 225–250 g were housed in the animal facility, Biomedical & Biological Science Research Building, University of Kentucky and allowed ad libitum access to water and food. All animal procedures were approved by the Institutional Animal Care and Use Committee, University of Kentucky and according to NIH guidelines. Prior to surgeries all the animals were randomly assigned into different experimental groups such that on any given day the surgeon and person administering drug or vehicle were blinded to treatment. Rats were anesthetized with Ketamine (80 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine (10 mg/kg, Lloyd Laboratories, Shenandoah, IA). A dorsal laminectomy was performed at the 12th thoracic vertebra to expose the first and second lumbar (L1/L2) spinal cord levels as described earlier (Patel et al., 2012), after which spinal cord contusions (250 kdyn) were performed with an Infinite Horizon impactor device (PSI, Lexington, KY). After injury the wounds were irrigated with saline, the muscles sutured together in layers with 3-0 Vicryl (Ethicon, Inc., Somerville, NJ), and the skin openings closed with wound clips (Stoelting Co., Wood Dale, IL). Hydrogen peroxide and betadine were used to clean the wound area and animals injected (s.c.) with pre-warmed lactated Ringer's solution (10 ml split into 2 sites bilaterally) and Cefazolin (33.3 mg/kg) before the rats were returned to their cages with food and water ad libitum. As soon as the rats regained consciousness, Buprenorphine-HCl (0.03 mg/kg; Reckitt Benckiser Pharmaceuticals Inc. Richmond, VA) was administered (s.c.) every 12 h for either 24 h (mitochondrial experiments) or 72 h (long-term behavioral experiments). Injured rats used for acute mitochondrial OCR (24 h survival times) were administered (i.p.) either vehicle (saline) or NACA (75, 150, 300 or 600 mg/kg bodyweight; NACA was a gift from Sentient Life Sciences, New York, NY 10021) 15 min post-injury followed by a corresponding booster at 6 h. For assessment of activities of mitochondrial enzyme complexes, injured animals were injected (i.p.) with either vehicle or NACA (75 or 300 mg/kg bodyweight) 15 min post-injury followed by a corresponding booster at 6 h. For long-term behavioral assessments, injured rats were treated (i.p.) with either vehicle, 150 or 300 mg/kg body weight NACA at 15 min post-injury followed by immediate implantation (s.c.) of primed osmotic mini-pumps (Model 2ML1; Alzet, Cupertino, CA) to continuously deliver 150 or 300 mg/kg/day for 7 days post-injury; after which rats were anesthetized under isoflurane for pump removal. NACA used for the current study was provided by David Pharmaceuticals, New York, NY.

Administration of osmotic mini-pumps

For continuous delivery of NACA for glutathione and long-term behavioral assessments, injured rats were treated (i.p.) with either vehicle, 150 or 300 mg/kg body weight NACA at 15 min post-injury followed by immediate sterile implantation (s.c.) of primed osmotic mini-pumps (Model 2ML1; Alzet, Cupertino, CA) to continuously deliver 150 or 300 mg/kg/day for 24 h or 7 days post-injury. Briefly, a small subcutaneous pocket was created on the animals' back by carefully separating the skin from the fascia layer and an osmotic pump was inserted with the delivery port facing rostral towards the injury site. The muscle layers and skin openings were closed as described above. After the 7-day delivery period, rats were re-anesthetized under isoflurane for pump removal.

Isolation of total, synaptic and non-synaptic mitochondria from a single spinal cord segment

All three mitochondrial populations were isolated from a single spinal cord segment by modifying our earlier described methods (Patel et al., 2009; Sullivan et al., 2007). At 24 h post-injury, animals were decapitated and the thoraco-lumbar spinal cords from naïve as well as injured animals were rapidly dissected out and placed in an ice cold dissecting plate containing isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, and pH adjusted to 7.2 with KOH). The following protocol is described for each sample and the steps are repeated for each animal. A 1.5 cm spinal cord segment centered on injury site was homogenized in 2 ml of ice cold isolation buffer (with EGTA). The homogenate was

Table 1
Number of animals used for the study.

Outcome measure	Beginning number of animals	Number of experimental groups	Final number per group
Mitochondrial OCR (24 h)	24	6	4
Mitochondrial enzyme activity (24 h)	72	4	6[a]
GSH-bolus (24 h)	12	4	3
GSH-bolus + continuous (24 h)	12	3	4
Long-term behavior experiment 2	21	3	5–7[b]

[a] Mitochondria isolated from 3 spinal cord segments were pooled in order to get sufficient protein to measure activities of enzyme complexes.
[b] Due to post-surgical complications, one rat from vehicle-treated group died and data for 1 rat was excluded from data analysis due to >2 standard deviation difference from group mean in histological assessment using ANOVA and student Newman–Keuls post hoc analysis.

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