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Disrupted mitochondrial genes and inflammation following stroke

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ABSTRACT

Aims: Determine the subacute time course of mitochondria disruption, cell death, and inflammation in a rat model of unilateral motor cortical ischemic stroke.
Main methods: Rats received unilateral ischemia of the motor cortex and were tested on behavioral tasks to determine impairments. Animals were euthanized at 24 h, 72 h and 144 h and mRNA expression of key mitochondria proteins and indicators of inflammation, apoptosis and potential regenerative processes in ipsilesion cortex and striatum, using RT-qPCR. Mitochondrial proteins were examined at 144 h using immunoblot analysis.
Key findings: Rats with stroke induced-behavioral deficits had sustained, 144 h post-lesion, decreases in mitochondrial-encoded electron transport chain proteins NADH dehydrogenase subunit-1 and cytochrome *c* oxidase subunit-1 (mRNA and protein) and mitochondrial DNA content in perilesion motor and sensory cortex. Uncoupling-protein-2 gene expression, but not superoxide dismutase-2, remained elevated in ipsilateral cortex and striatum at this time. Cortical inflammatory cytokine, interleukin-6, was increased early and was followed by increased macrophage marker *F4/80* after stroke. Cleaved caspase-3 activation was elevated in cortex and growth associated protein-43 was elevated in the cortex and striatum six days post-lesion.
Significance: We identified a relationship between three disrupted pathways, (1) sustained loss of mitochondrial proteins and mitochondrial DNA copy number in the cortex linked to decreased mitochondrial gene transcription; (2) early inflammatory response mediated by interleukin- 6 followed by macrophages; (3) apoptosis in conjunction with the activation of regenerative pathways. The stroke-induced spatial and temporal profiles lay the foundation to target pharmacological therapeutics to these three pathways.

1. Introduction

Stroke is the fourth leading cause of death and the leading cause of long-term disability in the US [1]. Annually, stroke is responsible for 130,000 deaths with an estimated cost of 34 billion dollars for medications, health care, and disability services [2,3]. Ischemic strokes make up 87% of all strokes [4] and treatment for ischemic stroke patients is limited. Many studies have focused on neuroprotective drugs that are administered prior to or within minutes to hours after stroke. While tissue plasminogen activator (TPA) has been found to be effective at reducing stroke-induced tissue loss, TPA has a small window of effectiveness (<4 h post-stroke) and can have severe consequences in hemorrhagic patients. Finally, only 25–46% of patients arrive at the hospital within 3 h after stroke warning signs [5–7]; therefore, there remains a need for treatment options beyond the first few hours after stroke.

Within minutes to hours after injury, apoptosis starts to occur, normally due to calcium influx and mitochondria dysfunction [8]. Degeneration of distal axons, also known as Wallerian degeneration occurs days to weeks following injury due to onset of deleterious metabolic pathways which leads to expansion of infarct size and worsening of clinical outcome. The area undergoing secondary injury that surrounds the core of the ischemic lesion is termed the penumbra and this peri-infarct tissue is clinically attractive due to the delayed onset of pathogenic mechanisms, which may be amenable to therapeutic interventions [9].

In addition to synthesizing ATP, the mitochondrion is also important in cell metabolism, calcium homeostasis, free radical production, and

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apoptosis [10–12]. During the secondary phase of ischemic injury, these mitochondria-dependent pathways are disrupted leading to increased reactive oxygen species, intracellular calcium and induction of pro-apoptotic cascades [8,13,14]. Thus, the development of pharmacological agents to promote recovery of mitochondria and ATP-dependent cellular functions may limit secondary neuronal damage in peri-infarct tissue.

Mitochondria abundance and the integrity of mitochondrial DNA (mtDNA) is disrupted following subacute brain insult [15] and is crucial for recovery of cellular function following ischemic injury [16,17]. Therefore, the aim of this study was to examine the subacute time course of mitochondria dysregulation by examining genes that encode for respiratory chain subunits and link these mitochondria changes to common pathological pathways such as neuroinflammation and cell death, following ET-1 induced stroke in the caudal forelimb area of the sensorimotor cortex (SMC) [18]. Because previous studies have shown that rescued function of SMC is dependent on preservation of the peri-infarct motor and sensory cortex and dorsal lateral striatum, we hypothesized that disruptions in cortical and striatal mitochondrial homeostasis in concert with neuroinflammation and cell death will result in impaired behavior outcomes. There are limited studies that explore the effect of mitochondrial dysregulation in the peri-infarct cortex and less is known about mitochondria function in brain regions interconnected via neural pathways, such as the striatum following a motor cortical stroke. Thus, this study will also elucidate the pattern of damage in the striatum to better understand secondary injury following ischemic stroke.

2. Materials and methods

2.1. Animals

Long Evans male rats (n = 86, 3–4 months old) received food and water ad libitum and were kept on a 12:12 hr light:dark cycle. Rats were randomly assigned to one of six groups that received either a sham or stroke procedure and were euthanized at one of three time points: 24 h (sham = 10; stroke = 10), 72 h (sham = 14; stroke = 22), or 144 h (sham = 10; stroke = 20). All animal protocols followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Medical University of South Carolina Animal Care and Use Committee.

2.2. Surgical procedures

Rats were anesthetized with ketamine (1.1 mg/kg I.P.) and Xylazine (0.7 mg/kg I.P.). Unilateral ischemic lesions were induced via ET-1 (American Peptide, Inc) applied to the cortical surface of the forelimb area of the SMC (Fl-SMC). Briefly, a craniotomy was performed at 1.0 mm posterior and 2.0 mm anterior to bregma and 3.0–5.0 mm lateral to midline and dura was gently retracted. ET-1 was applied on the brain surface at approximately 1 μL/min, with a 2 min wait between applications using a total of 4 μL. After the final 1ul of ET-1, the brain was left undisturbed for 5 min and then the craniotomy was covered with gel film (Invotec International) and dental acrylic. The stroke hemisphere was randomized. Sham animals had all procedures up to craniotomy. All animals received buprenorphine (0.5 mg/kg S.C.) prior to incision for pain.

2.3. Ladder task

To assess ischemia-induced impairments of forelimb function and compare these to mitochondria homeostasis markers, all animals were tested on the ladder task on days 0, 1, 3, and 6. The ladder task was used to assess coordinated forelimb use, stepping accuracy, and limb placement and is sensitive to motor cortex damage [19]. The ladder apparatus is made of two plexiglass walls, with 3 mm diameter pegs spaced 1 cm apart from each other. The ladder is raised ~20 cm off the ground with a neutral start cage and the animal's home cage at the end. Through slow-motion video replay, all forelimb placements were qualitatively scored on a 0–6 rating scale over three trials (three traverses across the ladder). A perfectly placed limb received a score of 6. Errors were scored as follows: 0 = limb missed the ladder rung and the limb fell through the rungs; 1 = the limb was placed the limb but when weight bearing either fell (score of 1) or slipped (score of 2) [19]. Percent errors was calculated as: sum of errors (0 + 1 + 2)/(total steps) per test day.

2.4. Tissue collection

Animals were deeply anesthetized with Euthasol (0.1 mg/kg) and brains were removed to obtain fresh tissue punches from the ipsilesional sensory and motor cortex and the striatum. Samples were taken medial and anterior to the injury based on specific lesions and anatomical observation, no tissue was sampled that contained the lesion core. Tissue was placed on dry ice to preserve mRNA and protein levels. Samples remained in a −80 freezer until RNA isolation or western blot analysis was performed. We choose to investigate the motor and sensory cortex that did not include the lesion core because these areas are highly connected to the primary area of injury, undergo secondary degeneration, and are important for recovery of sensorimotor function following caudal forelimb injuries and thus are targets for future intervention [20–22]. Additionally, we examined the entire striatum because it is known to undergo functional plasticity following stroke and is thought to be critical to intervention [23]. Investigation of the dorsal-lateral striatum would have provided more targeted information.

2.5. RNA isolation and real-time PCR

Total RNA was extracted from cortex and striatum using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific) with 0.5–1 μg of RNA. 5 μL of cDNA template was used to amplify PCR products using 2× Maxima SYBR green qPCR master mix (Thermo Fisher Scientific). The primer sequences used in

Table 1
Primer pairs used for qRT-PCR. List of rat primer pairs used in qRT-PCR.

Gene	Primer sequence	
PGC-lα	Sense anti sense	5′-AGGAAATCCGAGCGGAGCTG A-3′ 5′-GCAAGAAGGCGAC AC ATCG AA-3:
NDUFS1	Sense anti sense	5′-AGATGATTTGGG AAC AACGG-3′ 5′-TAAGGCTTAGAGGT T AGGGC-3′
COX1	Sense anti sense	5′-CCTGAGCAGGAAT AGT AGGG-3′ 5′-AGTGGTACAAGTCAGTTCCC-3′
ND1	Sense anti sense	3^1-T GAAT CCGAGCATCCT ACC-3^1 3′-ATTCCTGCTAGGAAAATTGG-3′
SOD2	Sense anti sense	5^1-C AAGGGAG AT G T T AC AAC TC AGG-3: 5′-CTTAGGGCTCAGGTTTGTCCA-3′
UCP2	Sense anti sense	5^1-GAGATACCAGAGCACTGTCG-3^5 5^1-GCT C AGT AC AGTT G AC A AT GG-3′
F4/80	Sense anti sense	5′-TCCTCTTCTGGGGCTTCAGT-3′ 5^1-CCA 11GC1GGGCAGAAAACC-3′
IL-6	Sense anti sense	5′-TTCAGAGCAAT ACT G AAACCC-3^5 5′-GATGGTCTTGGTCCTTAGCC-3′
Tubulin	Sense anti sense	5′-CTCTCTGTCGACTACGG AAAG-3′ 5′-TGGTGAGGATGGAATTGTAGG-3′
Actin	Sense anti sense	5′-TAAGGAAC AACCCAGC ATCC-3^5 5′ -CAGTGAGGCCAGGAT AG AGC-3^5