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Human genome comparison of paretic and nonparetic vastus lateralis muscle in patients with hemiparetic stroke

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Abstract—Hemiparetic stroke leads to major skeletal muscle abnormalities, as illustrated by paretic leg atrophy, weakness, and spasticity. Furthermore, the hemiparetic limb muscle shifts to a fast-twitch muscle fiber phenotype with anaerobic metabolism. This study investigated whether skeletal muscle genes were altered in chronic hemiparetic stroke. The nonparetic leg muscle served as an internal control. We used Affymetrix microarray analysis to survey gene expression differences between paretic and nonparetic vastus lateralis muscle punch biopsies from 10 subjects with chronic hemiparetic stroke. Stroke latency was greater than 6 months. We found that 116 genes were significantly altered between the paretic and nonparetic vastus lateralis muscles. These gene differences were consistent with reported differences after stroke in areas such as injury and inflammation markers, the myosin heavy chain profile, and high prevalence of impaired glucose tolerance and type 2 diabetes. Furthermore, while many other families of genes were altered, the gene families with the most genes altered included inflammation, cell cycle regulation, signal transduction, metabolism, and muscle contractile protein genes. This study is an early step toward identification of specific gene regulatory pathways that might lead to these differences, propagate disability, and increase vascular disease risk.

Key words: cell cycle, gene expression, hemiparetic stroke, inflammation, metabolism, microarray, muscle contraction, rehabilitation, skeletal muscle, transcription factors, vastus lateralis.

INTRODUCTION

Stroke is a leading cause of disability in the United States. More than two-thirds of stroke survivors have chronic functional deficits [1]. Hemiparesis is the most common neurological sequela. Skeletal muscle has recently been recognized as a site of secondary biological change after stroke that may propagate the functional decline and contribute to the increased risk of insulin resistance, cardiovascular disease, and recurrent stroke [2–7]. A number of pathophysiological mechanisms underlie

Abbreviations: DNA = deoxyribonucleic acid, G_1 = Gap 1, MHC = myosin heavy chain, MLC = myosin light chain, MYH = myosin heavy polypeptide, NIA = National Institute on Aging, RNA = ribonucleic acid, S = Synthesis, SCI = spinal cord injury, SEM = standard error of the mean, SF-36 = 36-item Short Form (Health Survey), SSWS = self-selected walking speed, TNF-α = tumor necrosis factor-α, VA = Department of Veterans Affairs, VL = vastus lateralis, VO_2 = oxygen consumption.

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the biological changes in muscle in stroke survivors. Stroke alters the motor neuron activation of muscles on the paretic side. After stroke, individuals have a more sedentary lifestyle and an altered gait pattern with reduced weight bearing, which also affect the skeletal muscle. Alterations in skeletal muscle are pronounced on the hemiparetic side after stroke and include gross muscular atrophy, increased intramuscular fat, and a shift to a fast myosin heavy chain (MHC) phenotype with reduced muscle oxidative capacity [8–11]. These muscle abnormalities after stroke have clear implications, since fast-twitch muscle fibers are more fatigable and have anaerobic metabolism [12]. The loss of slow-twitch fibers that take up glucose in response to insulin is relevant to the high prevalence of insulin resistance in stroke survivors [13]. In addition, expression of tumor necrosis factor alpha (TNF-α), an inflammatory cytokine, is elevated in the hemiparetic leg muscle compared with the nonparetic leg muscle [14]. Whether broader inflammatory pathway activation is present with subsequent downstream impact on muscle cell cycle, protein synthesis, or metabolic function is unknown.

The molecular mechanisms responsible for muscle atrophy, phenotype shift, and altered metabolism after stroke are not known. Stroke subjects offer an interesting perspective for studying the biological changes in hemiparetic muscle, since each subject's nonparetic leg muscle serves as his or her own internal control. The use of bilateral muscle samples eliminates individual genetic variation, which is a considerable limitation of microarray analysis of human tissues. The vastus lateralis (VL) muscle was chosen since all our subjects with hemiparetic stroke have quadriceps weakness and the majority have evidence of spasticity of knee extension. Furthermore, the quadriceps muscle is traditionally examined in studies of aging, diabetes, and exercise interventions [14–15]. This study used Affymetrix deoxyribonucleic acid (DNA) microarray analysis to investigate global gene expression changes underlying the biological changes of muscle structure and metabolic function in paretic and nonparetic VL muscle from subjects with chronic stroke.

METHODS

Men and women (n = 10) 50 to 80 years old with residual mild-moderate hemiparetic gait from an ischemic stroke (>6 months poststroke onset, range 7–156 months poststroke) participated in this study of altered skeletal muscle gene expression after stroke. A mild-moderate hemiparetic gait was defined by a clear gait asymmetry with preserved ability to ambulate with or without an assistive device, such as a cane, walker, or ankle-foot orthosis. All conventional rehabilitation therapy was completed more than 12 weeks before study entry, and neurological deficits were stable for more than 8 weeks.

The chronic phase of stroke was selected to ensure stability of the residual hemiparetic gait deficits and avoid any potential confounders of early neurological recovery or intercurrent rehabilitation therapy on skeletal muscle structure and function. Furthermore, the chronic stroke phase was chosen because in spinal cord injury (SCI), another upper motor neuron disorder, alterations in skeletal muscle contractile proteins have been shown to progress over many months after injury [16].

From the larger chronic stroke population participating in our rehabilitation programs (N = 72), we randomly selected 10 participants who had given consent for bilateral VL muscle biopsies. Exclusion criteria included medical conditions that limited mobility and participation in exercise programs, recent (<3 weeks) infection or inflammation, anti-inflammatory medications, anticoagulation, or known muscle disease. Individuals provided written informed consent. For each subject, we administered a comprehensive medical history, routine medical and neurological examinations, and questionnaires to estimate stroke disease burden. Cardiovascular fitness levels (peak oxygen consumption [VO_2] levels) were measured by open-circuit spirometry [17]. Functional and mobility testing included home and community ambulatory activity recorded by a diary and a computerized step activity monitor for 48 hours and the mean of three 10-meter walks that used self-selected walking speed (SSWS) as an index of gait deficit severity [17–18]. Individuals were categorized as having normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes mellitus according to medical history and prescribed medications, fasting glucose levels, and an oral glucose tolerance test (in nondiabetic participants). The University of Maryland Institutional Review Board approved all aspects of this study.

Bilateral VL muscle biopsies were obtained under local anesthesia with a 5 mm Bergström needle (Stille-Werner; St. Paul, Minnesota). VL samples were immediately frozen in liquid nitrogen and stored in a –80 °C freezer until assay. We isolated total ribonucleic acid

(RNA) from VL muscle with TRIzol® Reagent (Invitrogen; Carlsbad, California, catalog number 15596-026) using previously described methods [19]. The RNA was further purified with Qiagen's RNeasy Mini Kit and RNase-free DNase Set (Valencia, California, catalog numbers 74104 and 79254, respectively) according to the manufacturer's instructions. RNA purity and integrity were determined by the A260/A280 ratio and 28S/18S ratio, respectively. Purified RNA was provided to Gene Logic (Gaithersburg, Maryland), where purity and integrity were reverified by the A260/A280 ratio as well as analysis by an Agilent Bioanalyzer (Palo Alto, California, part number 5067-1511). RNA that was deemed pure was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Santa Clara, California, part number 900470) and differential gene expression was analyzed with Gene Logic software.

Statistical analysis was performed with Genesis Enterprise System® software (Gene Logic). This analysis created an *F*-score for each gene measured to determine significance ($p < 0.05$). For a gene to be considered significantly different between the paretic and nonparetic VL muscles, a minimum of 8 of the 10 patients in the study had to unidirectionally differentially express a difference for the gene of interest. Additionally, only genes that had expression differences of at least +1.5-fold between the paretic and nonparetic VL muscle samples were reported.

RESULTS

Subject descriptive data are presented in the **Table**. Participant age ranged from 50 to 80 years. The mean ± standard error of the mean (SEM) stroke onset latency was 59.1 ± 18.0 months. Seven individuals required a single point cane, two required a quad cane, none required a walker, and four required an ankle-foot orthosis. The subjects' SSWSs for the timed 30-meter walks with their usual assistive devices were slow (mean ± SEM 0.51 ± 0.10 m/s, range 0.45–2.34 m/s). Furthermore, cardiovascular fitness levels (peak VO_2) were significantly reduced in this population (mean ± SEM 13.7 ± 1.3 mL/kg/min, range 9.1–19.0 mL/kg/min). This functional data for stroke participants demonstrated how the disability of stroke affected their activities and performance, such as walking speed and fitness level. We administered the 36-item Short Form (SF-36) Health Survey to all participants to estimate their perception of disease burden. The participants' mean ± SEM SF-36 Health Survey physical health score was 50 ± 4.7 and mental health score was 76 ± 2.9. For the subset of eligible individuals who gave consent for this muscle biopsy study, no significant clinical (peak VO_2, walking speed, activity level) or demographic (age, sex, body mass index) differences were found compared with the larger cohort of individuals with chronic stroke participating in our exercise and rehabilitation programs. The **Table** includes demographic and clinical data demonstrating that the random population selected for muscle biopsies was the "same" as our overall stroke cohort population.

RNA from all subjects was deemed pure as evidenced by an optical density ratio higher than 1.8. This ratio was confirmed by Gene Logic, as well. The RNA concentrations did not differ between paretic and nonparetic VL muscle (all values are presented as mean ± SEM unless otherwise noted). The RNA concentration was 803.5 ± 22 μg/mL for the paretic VL and 911.8 ± 45 μg/mL for the nonparetic VL. In this experiment, 116 genes were significantly altered between paretic and nonparetic VL muscle. The **Appendix** contains a list of identifiable genes significantly altered; genes are shown grouped by function (available online only at http://www.rehab.research.va.gov). The genes with differential expression included genes related to inflammation, cell cycle regulation, mitogenesis, signal transduction pathways, metabolism, and muscle contractile proteins.

DISCUSSION AND CONCLUSIONS

This is the first known study to directly examine global gene transcript levels after a hemiparetic stroke. The major finding of this study is the identification of gene expression differences between the paretic and nonparetic VL muscles in chronic stroke survivors. We found substantial interlimb differences in a number of key molecular pathways that likely influence the fundamental structure, metabolism, performance, and regenerative potential of skeletal muscle tissue after stroke. **Figure 1** illustrates several pathophysiological factors that could contribute to the biological alterations in skeletal muscle after stroke. These factors include the abnormal neural activation of the paretic limb that causes weakness and spasticity after the stroke, relative disuse and deconditioning, and aging. An understanding of how stroke alters skeletal

Table.
Data for all subjects participating in larger hemiparetic stroke exercise study and for random subset of subjects who also consented to vastus lateralis (VL) biopsy. Spasticity was measured with modified Ashworth scale. Numbers (0/1/2/3) for spasticity represent number of subjects with spasticity ratings of 0 (normal), 1 (mild increase in tone), 2 (marked increased tone but able to move limb easily), or 3 (passive limb movement difficult because of increased tone). Steps were recorded for 48 hours with computerized step activity monitor (SAM). Self-selected walking speed (SWSS) was determined by timed 10-meter walk. Impaired glucose tolerance or type 2 diabetes mellitus was defined by known diabetes in medical history or medication profile, fasting glucose level, and 2-hour oral glucose tolerance test. Data presented as mean ± standard error of the mean unless otherwise noted.

Characteristic	VL Gene Array Study (*n* = 10)	Hemiparetic Stroke Exercise Study (*N* = 72)
Age (yr)	63.8 ± 2.9	63.6 ± 1.1
Male (%)	50	46
Latency (mo)	59.1 ± 18.0	50.8 ± 5.9
Left-Hemisphere Stroke (%)	40	50
Spasticity Rating 0/1/2/3 (No.)	5/3/1/1	35/20/14/3
Body Mass Index (kg/m^2)	27.0 ± 1.4	28.6 ± 2.7
VO_2 Peak (mL/kg/min)	13.7 ± 1.3	12.8 ± 0.5
SAM (steps/48 h)	4,335 ± 723	4,038 ± 362
SSWS (m/s)	0.51 ± 0.10	0.52 ± 0.03
Impaired Glucose Tolerance or Diabetes (%)	70	70

VO_2 = oxygen consumption.

muscle is important for recognizing how these changes contribute to the disability of stroke. Results of this study might lead to the development of new pharmacological or intervention strategies that improve muscle structure and metabolic function in stroke survivors.

Several major gene categories or clusters are differentially expressed between the paretic and nonparetic leg muscles, including genes that regulate muscle metabolism, contractile proteins, cell cycle progression, mitogenesis and growth factors, metabolism, inflammation, and signal transduction pathways. **Figure 2** shows the number of genes reported as significantly altered in each gene cluster and whether expression was higher in the paretic or nonparetic limb. Metabolism is one of the major gene clusters altered in the paretic leg muscle after stroke. Generally, slow-twitch muscle fibers have greater oxidative metabolism and fast-twitch fibers have greater glycolytic metabolism. In the current study, two oxidative enzyme genes were significantly down-regulated in the paretic leg. These oxidative genes are 3-oxoacid coenzyme A transferase 1 (*OXCT1*) and 3-hydroxybutyrate dehydrogenase, type 1 (*BDH1*) [20]. In addition, transcript

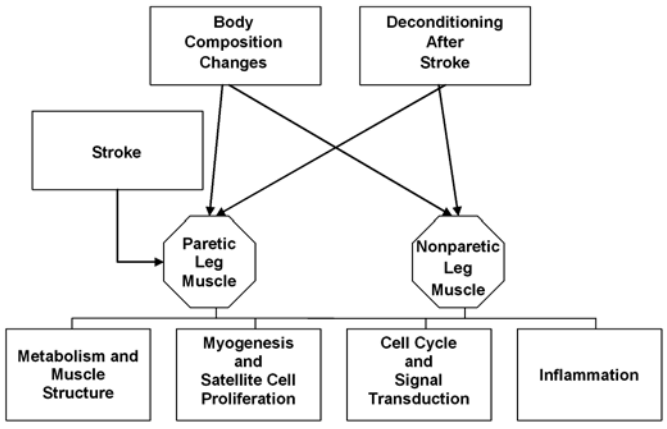

Figure 1.
Proposed influences of stroke on skeletal muscle and possible gene clusters induced by these muscle changes.

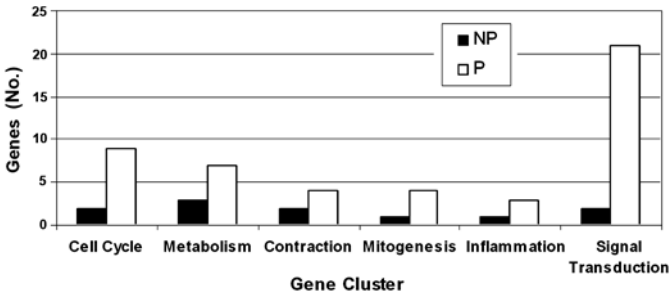

Figure 2.
Genes reported as significantly changed, shown grouped by cluster. Genes in nonparetic (NP) bars had lower expression in paretic (P) than NP vastus lateralis (VL) muscle, while genes in P bars had higher expression in P than NP VL muscle.

levels for glycerol-3-phosphate dehydrogenase 2 (*GPD2*), which is integral to muscle glycolysis, are significantly elevated in the paretic compared with the nonparetic leg. These findings are consistent with evidence based on muscle MHC isoform profiles that show that paretic leg skeletal muscle shifts to fast-twitch muscle fibers with increased reliance on anaerobic metabolism during single-leg exercise after stroke [3,12]. Prior muscle biology studies have demonstrated plasticity of muscle fiber composition and enzymatic activity that depend on the specific task requirements of individual muscles [21]; physical activity level [22]; and stimulus applied to the muscle, such as electrical stimulation [20]. Future studies should assess whether exercise or other rehabilitation strategies can modify these changes in gene expression in the hemiparetic leg muscle.

Changes in muscle contractile protein gene expression could have great consequences for muscle performance after stroke. Gross muscular atrophy predicts reduced cardiovascular fitness levels after stroke [23]. The shift to a fast MHC isoform in the hemiparetic leg is inversely related to a neurological deficit indexed by walking speed; individuals with the greatest shift in fast MHC isoforms had the slowest gait speeds [3]. Physical inactivity or detraining and muscle unloading have been previously reported to lead to a fiber type transition from slow oxidative to fast nonoxidative fibers [24–26]. In a nondisabled untrained person, the VL muscle is composed of various MHC isoforms: approximately 40 percent MHC I, 40 percent MHC IIa, and 20 percent MHC IIx muscle fibers. Chronic stroke produces more profound shifts in the fast MHC profile in skeletal muscle with 65 percent fast MHC fibers [3]. The current study found significant upregulation of embryonic myosin heavy polypeptide (MYH) isoform genes in the paretic leg. The *MYH3* and *MYH8* genes are up-regulated in the paretic leg 4.3-fold and 3.7-fold, respectively. Gene expression changes for other muscle contractile proteins, as well. Myosin regulatory light chains regulate the kinetics of force transmission of the specific MHC isoforms by modulating the binding of fast and slow myosin light chain (MLC) isoforms [27–28]. In the current study, the *MRLC3* and myosin light polypeptide 6B (*MYL6B*) genes were significantly down-regulated in the paretic leg after chronic stroke. Clearly, stroke leads to changes in gene expression for muscle contractile proteins, as evidenced by our MYH and MLC gene alterations. The factors regulating these changes in gene expression and the functional consequences are not known. MHC and MLC profiles can be altered by physical activity. In nondisabled volunteers, training can shift MHC to a slow profile with 60 percent MHC I, 30 percent MHC IIa, and 10 percent MHC IIx muscle fibers [29]. Similarly, functional electrical stimulation can shift the fast MHC isoform profile from SCI to a slow MHC profile [16,30]. Our laboratory is currently investigating whether an exercise intervention can reverse these MHC and MLC gene profiles in the paretic leg muscle after stroke.

We found that a number of genes that regulate myogenesis and satellite cell activation are altered in the paretic leg muscle after stroke. Upon muscle injury, satellite cells reenter the cell cycle, proliferate, and exit the cell cycle to differentiate into mature muscle fibers or proliferate to restore the satellite cell population [31–32]. Proliferating cells leave the cell cycle arrest (G_0 or resting) phase and enter the cell cycle at the Gap 1 (G_1) to Synthesis (S) phases through activation of the cyclin-dependent kinases signaling pathway. Muscle cell proliferation and differentiation are controlled through a series of myogenic regulatory factors. Consistent with these observations, we show that the paretic leg has increased gene expression of cyclin-dependent kinase inhibitor 1A (*CDKN1A*), which blocks progression from the G_1 to S phase. Growth arrest and DNA-damage-inducible 45 alpha (*GADD45a*), which is implicated in cell cycle arrest, apoptosis, and DNA repair [33], is upregulated in the paretic leg after stroke. We found that mammalian homologues of DNA mismatch repair (*MSH3*) genes are down-regulated in the paretic leg. Alterations of this gene are thought to contribute to cancer progression [34]. The paretic leg expressed increased gene levels of epidermal growth factor receptor (*ERBB3*), a tyrosine kinase that plays a critical role in the regulation of embryonic muscle development [35]. Additionally, myogenin, a gene involved in mature myofibril differentiation, exhibits elevated expression in the paretic leg. Platelet-derived growth factor C (*PDGF-C*) gene expression also is increased in the paretic compared with nonparetic leg in this study. This growth factor is often expressed in the myoblasts before they fuse into myotubes in developing embryonic muscle but not in adult skeletal muscle [36]. Altered signal transduction pathways and nuclear transcription regulation genes have previously been reported in the brain in neurodegeneration and after brain injuries [37–38]. Our present skeletal muscle study is in agreement, because several genes known to play a role in signal transduction or nuclear

transcription were reported as significantly altered. An understanding of the molecular regulation of satellite cell activation and myocyte differentiation after stroke could lead to development of new interventions that target satellite cells and thus improve muscle structure and performance after stroke.

Finally, a number of inflammatory genes were altered in chronic stroke muscle. We previously reported that TNF-α levels were significantly elevated in the VL muscles of the paretic compared with the nonparetic leg after stroke [14]. In the present study, we report elevated gene expression of a disintegrin and metalloprotease (*ADAM*) in the paretic leg muscle after stroke. *ADAM* proteins are important regulators of cellular adhesion and recognition, because they combine regulated proteolysis with modulation of cell adhesion to guide remodeling of the extracellular matrix along the migratory path [39]. In contrast, interleukin-17D, an inflammatory cytokine, is actually lower in the paretic leg than the nonparetic leg. On the basis of our study results, stroke appears to indeed activate specific inflammatory pathways and markers in the paretic leg. How these pathways interact and whether pharmaceuticals known to alter inflammatory cascades could limit inflammation remain to be elucidated.

The gene abnormalities noted between the paretic and nonparetic leg muscles in this study are likely secondary to altered neural innervation of muscle and disuse. Findings from this study are consistent with gene and protein changes after SCI. SCI causes a shift to a fast MHC isoform profile and a shift from oxidative to glycolytic muscle metabolism, similar to the skeletal muscle phenotype change after stroke [16,40–43]. The level of contribution from alterations in the neural activation pattern and from deconditioning remains to be elucidated. SCI results in an increased incidence of insulin resistance and diabetes, similar to the high prevalence of these conditions after stroke [5,7,44–45]. These results suggest that paresis resulting from an upper motor neuron lesion of stroke and a spinal cord lesion both induce similar changes in paretic muscles. In other Affymetrix studies probing global gene expression in skeletal muscle, common themes of altered gene expression occurred. Muscle unloading, sarcopenia of aging, and SCI result in upregulation of genes involved in the ubiquitin proteasome pathway and metallothione function [46–48]. In addition, muscle from older individuals have lower levels of genes encoding proteins that are involved in energy metabolism and mitochondrial protein synthesis compared with younger individuals. Skeletal muscle from older individuals also has elevated levels of genes involved in stress responses; hormone, cytokine, and growth factor signaling; cell cycle and apoptosis; and transcriptional regulation, consistent with dedifferentiation [44,48–49]. The β-adrenergic receptor agonists induce skeletal muscle hypertrophy and induce a different set of gene families. They induce genes associated with myocyte proliferation, myogenic differentiation, and/or recruitment of satellite cells into muscle fibers and genes involved in the initiation of translation to increase protein synthesis through upregulation of translational initiators [50–51]. Results from this study suggest that stroke induces changes in gene expression that parallel those in SCI, aging, and disuse but also have divergent gene family alteration from these other conditions.

Further investigations must confirm the magnitude of these gene changes by real-time reverse transcriptase polymerase chain reaction and determine whether these gene changes also alter protein levels and their activities in these muscles. One should note that stroke survivors likely take a wide variety of prescription medications, including aspirin, beta-blockers, and other antihypertensive and diabetic medications. Statins lower lipid profiles but also have anti-inflammatory benefits. These medications could potentially alter expression of specific genes. However, using each subject as his or her own control enabled us to limit interindividual dietary, medication, and genetic differences and target specific gene differences on the paretic and nonparetic sides. A limitation of this study is that it assumes that the changes in skeletal muscle are restricted to the paretic limb in stroke survivors. It does not account for more systemic changes that could affect gene expression even in the unaffected nonparetic leg muscle. Further studies using age-, disease-, and medication-matched nonstroke subjects are necessary to determine differences that may exist between both the paretic and nonparetic leg muscles and nonstroke control leg muscles. Additionally, stroke causes weakness and spasticity with increased muscular tone, which could both affect the magnitude of gene changes in the muscle. This study was not powered to determine the degree to which spasticity affects gene expression. Collectively, these data describe this population as being severely at risk for health-related detriments. In a patient with stroke, these muscle changes could affect muscle performance characteristics and cardiovascular fitness levels, as well as increase future risk of cardiovascular disease, diabetes, and metabolic syndrome.

Exercise has been shown to decrease the risk of cardiovascular disease, diabetes, metabolic syndrome, and functional decline [52]. Exercise can shift muscle fibers to a slow MHC phenotype and improve insulin sensitivity. Through insights from these genetic alterations in the paretic compared with the nonparetic leg muscles after stroke, we may develop therapeutic interventions that modify these altered gene expressions. Possible strategies could include pharmaceuticals [50–51], functional electrical stimulation [30,53], and aerobic or resistive exercise training. The beneficial effects of aerobic exercise training have been demonstrated in stroke [17,54–56] and SCI rehabilitation programs [57–60].

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