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Brief Report

Aging-related Increase in Rho Kinase Activity in the Nigral Region Is Counteracted by Physical Exercise

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Abstract

Abnormal activation of the RhoA/Rho kinase (ROCK) pathway plays a pivotal role in neuroinflammatory and pro-oxidative responses, axonal retraction, and apoptosis. We observed increased expression of RhoA, ROCK II, and ROCK activity in the brain of aged rats, particularly in the substantia nigra. Increased ROCK activity may enhance major mechanisms responsible for aging-related neurodegeneration, thus representing a major factor in the vulnerability of dopaminergic neurons to damage. We also observed that physical exercise decreased ROCK activation in aged rats. This suggests that decreased ROCK activation plays an important role in the neuroprotective effects of exercise observed in several previous studies. Furthermore, the present results suggest that ROCK inhibitors may constitute an effective neuroprotective strategy against aging-related risk of dopaminergic degeneration and possibly against other aging-related neurodegenerative processes.

Keywords: Aged—Neuroinflammation—Neuroprotection—Parkinson—ROCK

Aging is one of the most significant risk factors for the development of neurodegenerative diseases such as Parkinson's disease (PD) as a result of the increased vulnerability of neurons to damage (1,2). Several studies involving different tissues have shown that normal aging is associated with a proinflammatory, pro-oxidant state that may favor an exaggerated response to injury and degenerative diseases (3). Aged brains show a decreased ability to rescue damaged neurons and terminals or to be reinnervated by grafted neurons, which is generally related to the existence of a hostile environment in the aged brain (4). The mechanisms involved in creating this hostile environment are largely unknown. However, some of the mechanisms are counteracted by appropriate neuroprotective therapies or by physical exercise (5–7). To intervene in brain aging, we must first identify major factors that underlie it.

Abnormal activation of the RhoA/Rho kinase (ROCK) pathway has been observed in animal models of several brain diseases (8). It has been shown that ROCK plays a pivotal role in the microglial

neuroinflammatory response (9,10), and neuroinflammation plays a major role in the above-mentioned diseases (11), and in aging-related vulnerability of neurons to degeneration (1,3) and aging-related decrease in ability to rescue damaged neurons (12). ROCK activation is involved in axonal collapse and retraction, and ROCK inhibition may induce therapeutic effects by axon-stabilizing functions and promotion of neurite outgrowth (13). ROCK inhibition has also been observed to induce antiapoptotic effects and to have a beneficial influence on neuron survival (14). In Parkinson's disease models, recent studies carried out in our and other laboratories have shown increased levels of ROCK activity in the substantia nigra compared to normal controls not treated with dopaminergic neurotoxins, and that ROCK inhibition protects against dopaminergic neuron death and axonal retraction induced by dopaminergic neurotoxins (15). However, it is not known whether ROCK activation is enhanced in the brain (and the nigral region in particular) of aged animals, which could mediate the aging-related higher neuron vulnerability to damage, or whether

ROCK inhibition is involved in the beneficial effects of physical exercise.

Methods

Male adult Sprague-Dawley rats were divided into two groups. Rats in group A were young adults (3 months old; $n = 7$) and rats in group B were aged rats (18 months old; $n = 14$). The rats in group B were randomly assigned to exercise (treadmill running; $n = 7$) or no exercise ($n = 7$) subgroups. Nonexercised rats in groups A and B were subjected to the same handling than exercised rats except running during the 4-week training period (see later). A straight 2-lane treadmill (14 cm wide, 50 cm long) connected to a personal computer for system control and data management (CT-2 treadmill system, Columbus Instruments) was used for exercise. During a 2-day pretraining period (30 minutes/day at 6–10 m/min), rats that failed to complete the exercise session (ie, nonrunners) were excluded from the study [16]. Then the exercised rats were trained to run on the treadmill over 4 consecutive weeks (one 30-min session per day at 17 m/min, 5 days a week). Forty-eight hours after the last exercise session, exercised and nonexercised rats were killed and the area of the substantia nigra in the right and left ventral mesencephalon was dissected and processed for Western blot, real-time quantitative reverse-transcription polymerase chain reaction, and ROCK activity. All experiments were carried out in accordance with Directive 2010/63/EU and Directive 86/609/EEC and were approved by the corresponding committee at the University of Santiago de Compostela.

For Western blot, tissue was homogenized, processed, and transferred to nitrocellulose membranes, which were incubated overnight with primary antibodies against RhoA (1:200; sc-179) and ROCK II (1:200; sc-1851) from Santa Cruz Biotechnology. The HRP-conjugated secondary antibodies used were goat anti-rabbit (PI32460, Thermo Scientific and Protein G (18–161, Upstate-Millipore). Blots were stripped and reprobed for anti-GAPDH (G9545, Sigma; 1:25000) as a loading control. The data were then expressed relative to the value obtained for the control young rats (100%) to counteract possible variability among batches (see [9] for details). The relative levels of RhoA and ROCK II messenger RNA (mRNA) were examined by real-time polymerase chain reaction. β-Actin was used as a housekeeping gene and was amplified in parallel with the genes of interest. The data were evaluated by the delta–delta Ct method ($2^{-\Delta\Delta Ct}$), where Ct is the cycle threshold. Gene expression was determined relative to that of the housekeeping transcripts. For each gene forward (F) and reverse (R) primers were designed using Beacon Designer software (Bio-Rad; see [9] for details). ROCK (ROCK II) activity was measured using a ROCK Activity Assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions. Each sample was assayed in duplicate, and phosphorylation activity was assessed by measuring the absorbance at 450 nm in an Infinite M200 multiwell plate reader (TECAN) (see [9] for details).

All data were obtained from at least three independent experiments and were expressed as means ± *SEM*. Multiple comparisons were analyzed by one-way analysis of variance followed by a post hoc Holm Sidak test. The normality of populations and homogeneity of variances were tested before each analysis of variance. Differences were considered significant at $p < .05$. Statistical analyses were carried out with SigmaStat 3.0 software from Jandel Scientific (San Rafael, CA).

Results

The Western blot studies revealed significantly higher expression of RhoA and ROCK II protein in aged sedentary rats than in young sedentary rats. However, in aged exercised rats, RhoA and ROCK II protein levels were significantly lower than in aged sedentary rats (Figure 1A and B). Aging also induced significant increases in RhoA and ROCK II mRNA levels in the ventral midbrain (ie, nigral region) of aged sedentary rats relative to the RhoA and ROCK II mRNA levels in young rats. RhoA and ROCK II mRNA levels were significantly lower in aged exercised rats than in aged sedentary rats (Figure 1C and D). Finally, ROCK activity was significantly higher in the nigral region of aged sedentary rats than in young sedentary rats. However, ROCK activity was significantly lower in aged exercised rats than in aged sedentary rats (Figure 1E).

Discussion

The study results show increased ROCK activity in the brain of aged rats, particularly in the substantia nigra region. Increased ROCK activity may enhance major mechanisms responsible for aging-related neurodegeneration (such as abnormal neuroinflammatory and pro-oxidative responses, axonal retraction, and apoptosis) and may thus be an important factor in the greater vulnerability of aged brain neurons to damage. Interestingly, we also observed that physical exercise decreased ROCK activation in aged rats. Such a decrease in ROCK activation may contribute to the neuroprotective effects of exercise observed in a number of previous studies.

It is known that ROCK is present in neurons and different types of glial cells. However, several studies have shown that ROCK is predominantly expressed in microglia as compared with neurons and other glial cells [9,17]. We also observed that activation of microglial ROCK play a major role in neuroinflammation and dopaminergic neurodegeneration, and that inhibition of microglial ROCK plays a crucial role in the neuroprotective effects of ROCK inhibitors on dopaminergic neurons [9,15,18]. Altogether suggests that the increase in ROCK activity observed in the present study in aged rats is mostly related to microglial ROCK activation. Several mechanisms appear to be involved in ROCK-induced neuron vulnerability and the neuroprotective effects induced by ROCK inhibitors (for review see [15]). Microglial ROCK has been shown to play a crucial role in these effects [9,18]. In the central nervous system, activation of microglial ROCK mediates at least three major components of the microglial inflammatory response. First, RhoA/ROCK is an important regulator of the actin cytoskeleton, which is particularly important for microglial migration and several changes involved in phagocytosis [9,19]. Second, ROCK interacts with NADPH-oxidase [10] and ROCK inhibitors suppress activation of NADPH-oxidase [20]. In microglia and other inflammatory cells, NADPH-oxidase produces high concentrations of reactive oxygen species that are released extracellularly [21]. Finally, ROCK enhances microglial release of inflammatory cytokines such as interleukin-β and tumor necrosis factor-α [22,23]. In neurons, ROCK activation has also been shown to be involved in axonal collapse and retraction in the presence of inhibitory conditions, through modulation of the myosin light chain, phosphorylation of LIM kinase, and other mechanisms [13,24]. The axon-stabilizing effect of ROCK inhibition in damaged neurons has also been suggested as a mechanism of neuronal protection [24], and this has recently been confirmed in dopaminergic neurons [18,25]. It has also been suggested that ROCK inhibition activates neuroprotective survival cascades in dopaminergic neurons
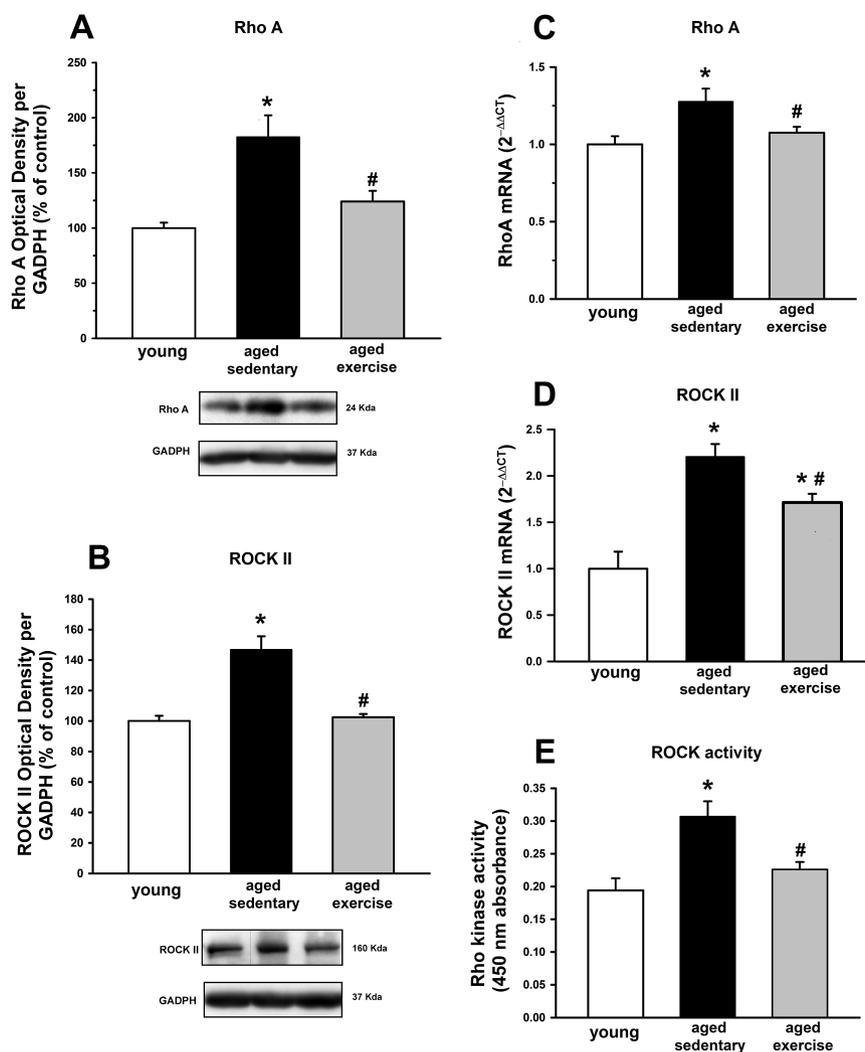
Figure 1. Western blot (WB; **A**, **B**), real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) (**C**, **D**) analysis of RhoA (**A**, **C**), and ROCK II (**B**, **D**) protein, and mRNA levels in aged rats relative to control rats (young sedentary rats). ROCK activity is shown in **E**. ROCK activation was significantly higher in aged rats, but this increase was significantly decreased by physical exercise. Protein expression was determined relative to that of the GAPDH band value and the expression of each gene was determined relative to the housekeeping transcripts (β-actin). The data were then expressed relative to control group values. Data are means ± *SEM*. $^*p$ < .05 relative to the young controls, $^#p$ < .05 relative to the aged sedentary group. One-way analysis of variance (ANOVA) and Holm Sidak post hoc test.

(25). A number of potential ROCK targets in apoptotic signaling have been suggested (14).

Several previous studies have revealed that physical exercise attenuates the aging-related decline in brain function (5,26). Exercise may act through multiple mechanisms, such as by enhancing the levels of several neurotrophic factors (6,7). However, decreased oxidative stress and inflammation (27,28) and antiapoptotic effects (29) have also been reported as major mechanisms responsible for the neuroprotective effects induced by exercise. Inhibition of the aging-related increase in ROCK activity may play a major role the anti-inflammatory, antioxidant, and antiapoptotic effects involved in neuroprotection by exercise. In conclusion, the present findings indicate that the age-related increase in ROCK activity observed in the substantia nigra of rats may be involved in the aging-related increase in vulnerability of dopaminergic neurons to damage, and that this can be attenuated by physical exercise. Furthermore, the present results suggest that ROCK inhibitors may constitute an effective neuroprotective strategy against the aging-related risk of dopaminergic degeneration and possibly other aging-related neurodegenerative processes.

Conflict of Interest

The authors have no competing interests to declare.

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