Induction of brain Nrf2-HO-1 pathway and antinociception after different physical training paradigms in mice

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ABSTRACT

Aim: Activation of the Nrf2-antioxidant response element signaling pathway is a major mechanism in the cellular defense against oxidative or electrophilic stress through conjugative reactions and by enhancing cellular antioxidant capacity. Although exercise training up-regulates antioxidant defenses system, while information regarding the intensity levels of physical exercise that acts on the cellular protection systems is limited.

Main methods: The present study evaluated the effects of different durations and intensities of physical exercise on the hippocampus, cortex and hypothalamus Nrf2 and HO-1 gene expression and related protein content and the nociception thresholds in adult C57Bl male mice. Exercise training consisted of daily running on a 10-lane rodent motor-driven treadmill for either 3 or 7 weeks at three different intensities. Pain responses were evaluated after exercise and in untrained mice by Von Frey hair test and cold plate test.

Key findings: This study confirmed that only vigorous and longer duration aerobic exercise increased Nrf2 protein level in the hippocampus and HO-1 protein level in the cortex and reduced pain perception. Mechanical and thermal hypoalgesia were only observed in exercise groups after 7 weeks of physical training.

Significance: The overall findings in this study confirm that only the long duration intensive forced exercise reduced inflammatory pain by induction of Nrf2/HO-1 antioxidant signaling pathway.

1. Introduction

The Nrf2 (nuclear factor E2-related factor 2)-antioxidant response element (ARE) signaling pathway is involved in the regulation of antioxidant, anti-inflammatory and cell survival genes [1]. This pathway is the most important antioxidant stress pathway identified to date [2]. A huge body of evidence confirms that Nrf2 is contributed to mitochondrial biogenesis and scavenge stressful conditions [3]. The Nrf2/ARE pathway is strongly linked to the connection between inflammation, apoptosis, autophagy, and mitochondrial functions [4]. In unstressed situations, Nrf2 is bound to the cytosolic inhibitor Kelch-like ECH-associated protein 1 (Keap1), which decreases Nrf2 protein level by proteasomal degradation [5]. Keap-1 is the main sensor during oxidative stress and an important driver of Nrf2 regulation in stressful and basal intracellular redox conditions [6]. Nrf2 is the necessary transcriptional inducer of phase II detoxifying proteins such as glutathione reductase, glutathione S transferase and heme oxygenase-1 (HO-1) [7]. In stressful conditions, Nrf2 translocates into the nucleus and by binding to ARE activates antioxidant machinery dependent molecules, resulting in upregulation antioxidant response genes such as of Nrf2-dependent heme oxygenase-1 (HO-1) [8].

This project visualized the consequences of physical effort on the Nrf2/ARE pathway. Many studies confirmed that exercise improves mental state, promotes neuronal survival and increases resistance against brain insults [9]. We focused on the Nrf2/HO-1 signaling pathway. Physical exercise modulated the anti-inflammatory responses observed in chronic pathologies and protect the body against inflammation [10]. This concept of the anti-inflammatory effects of physical training has gained increasing attention in recent years [11,12]. However, heterogeneity in the behavioral studies is important as results varied with the type of stimulus, duration and intensity of exercise [13,14] and the relationships between physical activity and endogenous pain inhibition are unclear [15]. Acute exercise leads to Nrf2/HO-1 activation [16], to increase activation of antioxidant promoter-dependent genes in wild-type mice subjected to treadmill exercise for two consecutive days and an increase in oxidative stress was
observed in Nrf2-deficient mice after exercise [17]. The occurrence of repeated painful stimuli during sustained physical exertion was never tested. Thus, rigorous and objective measures of pain were made, as it was important to design studies in which reproducible parameters were obtained. Physical exercise may modify the cerebral integration of nociceptive signals and reduce the local release of inflammatory mediators as interleukins, tumor necrosis factor-α, chemokines by activation of spinal microglia and secretion of anti-inflammatory cytokines such as IL-10 and IL-4 [18]. Accordingly, it has been described that these mediators contribute to the development of processes first in the medulla, which then persists and progresses to distant regions, such as the hypothalamus and periaqueductal grey area, both components of the nociceptive system [19]. It has also been described that the proinflammatory environment of the organism induced by stressors could interact along the gut-brain axis with brain function. This could also include changes in the plasticity of regions sensible to stressors including corticolimbic system, prefrontal cortex, amygdala and hippocampus, both regions which properties are altered with persistent pain [20,21]. Moreover, the generation of reactive oxygen species or reactive nitrogen species can alter the mitochondrial activity of both the hippocampus and prefrontal cortex, and cause subsequent suppression of synaptic plasticity [22]. The aim of this work was to observe the modifications of the transcription factors Nrf2 and HO-1 in three subfractions of the brain and to study simultaneously the influence of exercise duration and intensity on pain thresholds in mice using classical pain tests (Fig. 1).

2. Methods

2.1. Animals

All animals were carried out in accordance with “the guide for the care and use of laboratory animals”, Eighth edition (2011). All testing was conducted in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92-373 to FC) as well as with the European Directive 2010/63/EU. Nociceptive tests were carried out according to the ethical guidelines of the International Association for the Study of Pain [23]. Five-week-old male C57Bl mice (Janvier Labs, Le Genest sur l’Isle, France) weighing 19–21 g at the beginning of the study, were randomly assigned to five mice per cage in a temperature (21 ± 1 °C) controlled room with a 12 h light: 12 h dark cycle (lights on at 06:00 h). Food and water were available ad libitum except during behavioral observations. Cages were cleaned once a week. After training, animals were fasted overnight and sacrificed by cervical dislocation before tissues harvest.

2.2. Treadmill-running protocol

Exercise training consisted of running on a 10-lane rodent motor-driven treadmill (Medical Development®, Tecmachine®) during the day. Throughout the treadmill-running period, mice were stimulated to continue running by a soft air jet on the tail or hindquarters. To reduce their stress in response to this new environment, mice in the exercise-training groups were adapted to the rodent treadmill by walking for 20 min daily the week before starting the training program. After the period of adaptation, mice underwent an incremental exercise test to establish the maximal running speed for each mouse. Experiments were conducted in 2 cohorts: cohort 1: three weeks of exercise, cohort 2: seven weeks of exercise. In each cohort, four experimental groups of 10 mice were involved: three exercise groups and an untrained group. The exercise groups were subjected to low = (0.84 km·h⁻¹), intermediate velocity (1.2 km·h⁻¹) or high-speed velocity (1.4 km·h⁻¹), 5 times a week (duration of the session: 60 min). All mice in the exercise-training groups could fully comply with the training protocol.

2.3. Real-time PCR for gene expression analysis

Lysis of the different fractions of the brain was performed with TRIzol® (1 ml for cortex and 500 μl for hippocampi and hypothalamus), which allows cell lysis while maintaining and protecting the integrity of the RNA. After stirring in the presence of 200 μl of chloroform with 1 ml of TRIzol®, centrifugation at 12,000 × g for 15 min at +4 °C, makes it possible to separate the organic phase from the aqueous phase, which contains the RNAs. The aqueous phase was recovered and then the RNAs are precipitated by the addition of isopropanol, 0.5 ml of isopropanol with 1 ml of TRIzol®, and recovered by centrifugation at 12,000 × g for 15 min at 4 °C. The RNA pellets are then washed with 75% ethanol and then partially dried in the open air for 15 min. Finally, the RNA is solubilized in RNase-free water (60 μl for the cortex and 10 μl for the hippocampus) and frozen at −80 °C. The RNAs were then quantified by measuring the absorbance at 230, 260 and 280 nm. The reverse transcription reaction was performed on 1 μg of RNA using qScript cDNA SuperMix (Quanta, Biosciences). The samples are diluted in a final volume of 100 μl of water RNA.

For the study of the expression of the Nrf2 target genes (HO-1), the cDNAs obtained are analyzed by real-time quantitative PCR with reagents containing SYBR, which is a fluorescent label emitting a quantity of light proportional to its binding in the DNA. Double strand obtained by measuring the absorbance at 230, 260 and 280 nm. The reverse transcription reaction was performed on 1 μg of RNA using qScript cDNA SuperMix (Quanta, Biosciences). The samples are diluted in a final volume of 100 μl of water RNA.

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**Fig. 1.** Shows the schematic experimental design of the study. Mice were divided in four groups, No exercise, low intensity exercise (0.84 km·h⁻¹ corresponding to 50–55% of the VMA), intermediate intensity exercise (1.2 km·h⁻¹ corresponding to 65–70% of VMA) and high intensity exercise (1.4 km·h⁻¹ corresponding to 85% of the VMA). Pain behaviors were evaluated the second and third day of exercise termination. Mice were euthanized the next day, tissue were stored in −80 °C until analysis.
2.4. Western blot analyses

Samples for analysis were obtained upon sacrifice (each group, n = 5 mice). The brain three fractions (cortex, hippocampus, hypothalamus) were carefully removed and frozen at −80 °C. Standard technique was used to determine Nrf2 content as previously described [24]. Total protein concentration was evaluated using bicinchoninic acid assay (Sigma, St Louis, MO), and equal amounts of denatured proteins (60 μg) were loaded onto 10% SDS-PAGE gel and then transferred onto a PVDF membrane (Amersham Biosciences, Les Ulis, France). Membranes were incubated with the antibody raised against Nrf2 (H-300; Santa Cruz Biotechnology, Heidelberg, Germany) or HO-1 (ab13248; Abcam, Cambridge). After four washes in a 0.2% Tween TBS solution, the membrane was incubated for 45 min with a rabbit secondary antibody (SAR) for Nrf2 or with an anti-mouse secondary antibody (SAM) for HO-1, both are conjugated to peroxidase. Antibody against β-actine (C4, Santa Cruz Biotechnology) was used as a loading control. Quantification was determined by densitometry using densitometer associated with analytical software by the help of ChemiDoc (Biorad, Milan, Italie) and ImageLab® software (Biorad, Marnes-La-Coquette, France). Results were expressed as the fold induction that represented the ratio to the β-actin.

2.5. Evaluation of pain behaviors

To minimize treadmill influences on pain behaviors, nociception

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Fig. 2. Nrf2 protein levels in cortex, hippocampus and hypothalamus of mice submitted to treadmill exercise during 7 weeks at low, intermediate and high intensity exercise, the first group is the untrained control group. Nrf2 expression is analyzed by Western blot. Nrf2 intensity shows the fold relative change of Nrf2 protein translation to control. Results are expressed as means ± SD of four independent experiments *p < 0.05, **p < 0.01 vs untrained control group. Protein concentration intensity bar, A (Cortex), B (Hippocampus), C (Hypothalamus), D (related western blot image).
tests were performed 12 h after the last treadmill session. All behavioral tests were carried out by experimenter blindness.

- The von Frey test evaluated the intensity of the tactile reaction of the mice in response to a mechanical stimulus consisting in the application of a von Frey nylon filament which exerted a calibrated force on the plantar surface of the hind paws [25]. Mice were placed in elevated Plexiglas individual boxes (10 cm × 10 cm × 10 cm) with the wire-mesh floor, 30 min before starting the experiment. Mice were habituated to the test environment during 3 baseline sessions one week prior. Calibrated nylon filaments (Stoelting, Wood Dale, IL USA) of 3 different stimulus strengths, 0.16, 0.6 and 1.4 g, were applied in ascending order to the plantar surface of both the ipsilateral and contralateral hind paw (right and left) of all mice. For each filament, 5 repeated stimulations were applied lasting 2 s each, with a latency time between two successive stimulations of 3 s. The nociceptive response was characterized by a brisk paw withdrawal; the results were expressed as a number of paw withdrawals. A significant increase in withdrawal with the filaments 0.16 g and 0.6 g indicated mechanical tactile allodynia or mechanical tactile hyperalgesia with the filament 1.4 g.

- The cold-plate test: a cold stimulus was assessed using the cold-plate test as previously described [26]. The test used an electrically cooled and thermostatically controlled surface set to a temperature of 2.0 ± 0.1 °C (BIO-CHP, Bioseb, Vitrolles, France). Measurements were performed on both the ipsilateral and contralateral forepaws. The latency of withdrawal (in seconds) was measured in five-time trials each separated by a 15-min interval. Nociceptive response for the cold-plate test was expressed as the mean ± SEM of the latencies after exclusion of the maximum values.

2.6. Statistical analysis

Results were shown as mean ± SEM. Tactile and thermal hypersensitivity were indicated by a significant reduction in response thresholds relative to the untrained group values. The potential impact of training on nociception was assessed with two-way ANOVA followed by multiple comparison post hoc tests (Fisher’s LSD test least significant differences) with Prism.V.6 software (GraphPad Software Inc.; La Jolla, CA). Differences were considered statistically significant when p < 0.05.

3. Results

3.1. Nrf2

Fig. 2 showed the effect of the different exercise-induced Nrf2 modalities for the 7-week workout. A significant difference of Nrf2 protein concentration was observed only in the hippocampus (Fig. 2B). Nrf2 was detected for the group submitted to high intensity (1.2 km·h⁻¹) and intermediate intensity (1.2 km·h⁻¹) exercise, both compared to the untrained group (p = 0.008 and p = 0.040, respectively). In the cortex and hypothalamus, the physical effort did not modulate the expression of Nrf2 (Fig. 2A, C).

3.2. HO-1

Figs. 3 and 4 showed the effect of the different exercise-induced HO-1 modalities for the 7-week workout. No significant difference in gene expression was observed for HO-1 in all three brain areas (hypothalamus, hippocampus, and cortex) (Fig. 3A, B, C). But interestingly HO-1
Protein concentration was signifi-
cantly elevated in the cortex of inter-
mediate and high-intensity exercise group, compared to low intensity
and untrained group (Fig. 4A).

3.3. Cold plate test

- **3-weeks training** (Fig. 5A): Latency time to first licking of the paw
  \((p = 0.83)\) was not significantly changed in different exercise groups
  compared to the untrained group.
- **7-weeks training** (Fig. 5B): Elevation in latency time to first licking
  was observed. For the group subjected to a high-intensity exercise
  \((1.4 \text{ km·h}^{-1})\), the latency time was significantly higher, compared to
  the untrained group \((p = 0.004)\).

3.4. Von Frey hair test

- **3-weeks training** (Fig. 6A): No statistically significant difference was
  observed in numbers of paw withdrawals after 10 stimulations
  between different training groups and the untrained group, regard-
  less of the strength of filaments.
- **7-weeks training** (Fig. 6B): For the 1.4 g and the 0.6 g filaments,
  there was a significant decrease in the number of paw withdrawals
  in the high-speed group \((1.4 \text{ km·h}^{-1})\) compared to the untrained
  group \((p = 0.004\) and \(p = 0.012\), respectively).

4. Discussion

In our study, we confirmed that only high-intensity long duration
physical forced exercise increased the transcriptional activity of Nrf2
and HO-1 in different brain area and subsequently induced hypoalgesia
against mechanical and thermal painful stimuli. For the first time, we
evaluated the expression of aforementioned genes in the hippocampus,
cortex and hypothalamus and elevation of the nociception thresholds of
treadmill exercised mice. For a good precision, we used two types of
stimuli for pain measuring tests, the Von Frey test for mechanical al-
lodynia and cold plate test for thermal hyperalgesia evaluation. The
predominant elements of our study include the exercise-induced Nrf2 and HO-1 transcriptional activity elevation, and the exercise-induced hypoalgesia. This suggests that only the long duration intensive exercise operates as an inducer of hypoalgesia, via a pathway dependent on Nrf2-HO-1 signaling. Accordingly, a significant increase in HO-1 protein level was seen in intermediate- and high-intensity exercise groups, compared to the untrained group. The increase was significant for High: No exercise (p = 0.004).

A huge body of evidences confirm that (Nrf2)/HO-1/NAD(P)H:quinone oxidoreductase-1 (NQO1) signaling pathway has fruitful role as endogenous antioxidant defense system agents and plays a pivotal role in modulation/regulation cellular oxidation, cell defense, cell protection and reactive oxygen species (ROS) scavenging, involved in anti-nociceptive processes [27]. Some previous studies on animal and humans focused on the same elements but in different body tissues such as skeletal muscles [28], cardiomyocytes [29] and peripheral blood mononuclear cell (PBMC) [30], in different exercise training protocols. Exercise is capable to increase the level of some antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in different body tissues [31]. Evidence has implicated the exercise in the regulation of Nrf2 [32], but no research has been devoted to understanding its contribution to exercise-induced analgesia via this mechanism, found in our work in different brain regions. In a recent study, the expression of transcription factor Nrf2 was studied in peripheral blood mononuclear cells of patients diagnosed with chronic kidney disease, and a significant increase was observed after resistant physical exercise during hemodialysis [30]. In skeletal muscles of mice, an increase of Nrf2 transcriptional activity was described after a 6-week voluntary running wheel training protocol [28]. Some recent and old studies confirmed the exercise-induced elevation of Nrf2 in human peripheral arteries and striatum of mice brain [33,34] and demotion in skeletal muscles of a human with sedentary lifestyle [35]. It should be mentioned that the real mechanism that how exercise activates Nrf2 is not well known. It was recently observed that reactive oxygen species and nitric oxide produced in mice skeletal muscles during resistant acute exercise on the treadmill in different durations, would contribute to the activation of Nrf2 and mitochondrial antioxidant defense system upregulation [32,36].

Hippocampal down-regulation of the Nrf2-ARE signaling pathway induced local neuroinflammation, oxidative stress and cognitive

Fig. 5. Delay in seconds before licking of fore paws when mice were placed on a cold plate (+4 °C). Mice (n = 10) were previously submitted to treadmill exercise during 3 weeks (A) or 7 weeks (B) at low (0.84 km·h⁻¹ corresponding to 50–55% of the VMA), intermediate (1.2 km·h⁻¹ corresponding to 65–70% of VMA) and high intensity exercise (1.4 km·h⁻¹ corresponding to 85% of the VMA), the first group is the untrained control group. The increase was significant for High: No exercise (p = 0.004).

Fig. 6. Number of paw withdrawals out of five mechanical stimulations per hind paw using Von Frey filaments corresponding to three bending forces (0.16, 0.60 and 1.4 g). Mice (n = 10) are previously submitted to treadmill exercise during 3 weeks (A) or 7 weeks (B) at low (0.84 km·h⁻¹ corresponding to 50–55% of the VMA), intermediate (1.2 km·h⁻¹ corresponding to 65–70% of VMA) and high intensity exercise (1.4 km·h⁻¹ corresponding to 85% of the VMA), the first group is the untrained control group. The increase was significant for High 0.6 g: No exercise 0.6 g (p = 0.0073), Weak exercise 1.4 g: No exercise 1.4 g (p = 0.0118), Intermediate exercise 1.4 g: No exercise 1.4 g (p = 0.0112), High exercise 1.4 g: No exercise 1.4 g (p = 0.00427).
impairments [2]. These findings, along with our observation that exercise can increase the transcriptional activity of Nrf2 and HO-1 in the brain, suggest that Nrf2 participates in the initial signaling events that culminate in the expansion of antioxidant defense system and decreases inflammatory pain with exercise training. Concerning HO-1 expression, it can be induced by different transcription factors, such as Nrf2, Hypoxia-inducible factor-1 (HIF-1), Nuclear factor κB (NF-κB) and activating protein-1 (AP-1) [37]. Some studies confirm that HO-1 gene was also expressed by some signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 phosphate (PI3)/akt [38].

A major hurdle in investigating the mechanism, by which exercise may influence the inflammation and nociception via Nrf2-HO-1 signaling pathway, is the lack of knowledge on the molecular mechanism underlying the hippocampal Nrf2 expression and peripheral nociception. However, the effects produced by this training exercise protocol have not been evaluated before. Nrf2 expression level is more contributed to the changes in AMPK signaling, which has been confirmed to be more responsive to exercise duration rather than intensity (Morales-Alamo et al., 2016).

A wide range of evidence indicates that Nrf2 can control the different mechanisms involved in the inflammatory situation, oxidative stress, immune response and cartilage and bone metabolism [39]. Chung et al. hypothesized that exercise improves mitochondrial functions and reduces oxidative stress in the brains of patients with Parkinson disease [40]. A most recent study confirmed that down-regulation of Nrf2 could result in inhibition of chondrogenesis through apoptotic cell death [39,41]. Induction of HO-1 by Nrf2 activation can also play a role in its anti-inflammatory and chondroprotective effects as HO-1 is able to reduce NF-κB activity and inflammatory and degenerative mediators in chondrocytes, synoviocytes and osteoblasts [42–44].

In the light of this agreement, we found that exercise attenuated hyperalgesia and alldynia probably induced by inflammation. However, only a chronic forced and regular exercise can modify nociceptive thresholds compared to the untrained and low-intensity short-term exercise mice and can significantly increase the expression of HO-1 and Nrf2 in the brain. The alteration of alldynic and hyperalgesic nociception in the training group of our study may be contributed to the mechanism discussed in Clerigues et al. works [44]. The anti-alldynic and anti-hyperalgesic effect of HO-1 induction was also described in mice, using stimuli as von Frey, and hot plate test [27].

5. Conclusion

These findings indicated that the Nrf2-HO-1 signaling pathway activation by long duration and high-intensity exercise may increase the pain threshold. As oxidative stress is attributed to various diseases related to inflammatory cytokines induction and to hyperalgesia, evaluating the underlying molecular mechanism may have a potential therapeutic implication. There is limited information to lighten the triangle of physical exercise, Nrf2-HO-1 signaling pathway and inflammatory pain perception threshold measurement. Our work confirmed that this protocol of exercise can activate important transcriptional factors and induce related anti-oxidant genes in different brain regions. More studies are required to further elucidate the underlying mechanism of chronic intensive exercise-induced expression of Nrf2-HO-1 signaling pathway and anti-nociceptive effects.

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Compliance with ethical standards

The authors had no competing financial interest relevant to this article to disclose. This article does not contain any studies with human participants performed by any of the authors. All animals were carried out in accordance with “the guide for the care and use of laboratory animals”, Eight Edition (2011). Nociceptive tests were carried out according to the ethical guidelines of the International Association for the Study of Pain.

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