Molecular hydrogen reduces acute exercise-induced inflammatory and oxidative stress status

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Original article

Molecular hydrogen reduces acute exercise-induced inflammatory and oxidative stress status

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ABSTRACT

Physical exercise induces inflammatory and oxidative markers production in the skeletal muscle and this process is under the control of both endogenous and exogenous modulators. Recently, molecular hydrogen (H_2) has been described as a therapeutic gas able to reduced oxidative stress in a number of conditions. However, nothing is known about its putative role in the inflammatory and oxidative status during a session of acute physical exercise in sedentary rats. Therefore, we tested the hypothesis that H_2 attenuates both inflammation and oxidative stress induced by acute physical exercise. Rats ran at 80% of their maximum running velocity on a closed treadmill inhaling either the H_2 gas (2% H_2, 21% O_2, balanced with N_2) or the control gas (0% H_2, 21% O_2, balanced with N_2) and were euthanized immediately or 3 h after exercise. We assessed plasma levels of inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL) − 1β and IL-6] and oxidative markers [superoxide dismutase (SOD), thiobarbituric acid reactive species (TBARS) and nitrite/nitrate (NOx)]. In addition, we evaluated the phosphorylation status of intracellular signaling proteins [glycogen synthase kinase type 3 (GSK3α/β) and the cAMP responsive element binding protein (CREB)] that modulate several processes in the skeletal muscle during exercise, including changes in exercise-induced reactive oxygen species (ROS) production. As expected, physical exercise increased virtually all the analyzed parameters. In the running rats, H_2 blunted exercise-induced plasma inflammatory cytokines (TNF-α and IL-6) surges. Regarding the oxidative stress markers, H_2 caused further increases in exercise-induced SOD activity and attenuated the exercise-induced increases in TBARS 3 h after exercise. Moreover, GSK3α/β phosphorylation was not affected by exercise or H_2 inhalation. Otherwise, exercise caused an increased CREB phosphorylation which was attenuated by H_2. These data are consistent with the notion that H_2 plays a key role in decreasing exercise-induced inflammation, oxidative stress, and cellular stress.

1. Introduction

Physical exercise causes cellular and humoral changes that resemble, in some respects, the acute phase response to trauma and inflammation [1]. Accordingly, plasma levels of tumor necrosis factor-α (TNF-α) and interleukin (IL) − 6 (IL-6) are increased after physical exercise [2]. The mechanisms responsible for the production of exercise-induced cytokines are not completely known [2]. In addition, physical exercise increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the muscle accompanied by increases in plasma ROS and RNS [3].

It is known that regular exercise causes beneficial physiological adaptations in the body. Conversely, unaccustomed or exhaustive exercise can be harmful, causing excessive muscle damage, inflammation,

Abbreviations: CREB, cAMP responsive element binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3, glycogen synthase kinase type 3; H_2, molecular hydrogen; H_2O_2, hydrogen peroxide; IL-1β, interleukin-1β; IL-6, interleukin-6; NF-kB, nuclear factor transcription; NO, nitric oxide; NOx, nitrite/nitrate; $O_2^{\bullet -}$, superoxide radical; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species; TNF-α, tumor necrosis factor-α; Vmax, maximum running velocity

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and oxidative stress [4]. Oxidative stress is accepted as an imbalance between generation and removal of ROS/RNS by the antioxidative system [5]. Superoxide dismutase (SOD) is an essential antioxidant enzyme in the first-line defense mechanisms against oxidative stress [6]. SOD converts superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), which is subsequently converted to water by the catalase enzyme [7]. Measurements of SOD and thiobarbituric acid reactive species (TBARS - lipid peroxide indicator) [8] have been used as key oxidative stress markers [9,10]. In addition, nitric oxide [NO - a molecule that performs signaling and functional functions in the body [11]] is also involved in antioxidant mechanisms [12,13]. Moreover, oxidative stress can be attenuated by several processes and glycogen synthase kinase type 3 (GSK3) can play an important role in this scenario since its phosphorylation reduces mitochondrial production of ROS [14]. GSK3 is expressed ubiquitously in the cells of the body, presenting in two isoforms: GSK3α and GSK3β [15]. Several upstream proteins can phosphorylate GSK3, and GSK3 can phosphorylate downstream molecules such as the cAMP responsive element binding protein (CREB) [16]. Studies have shown that physical exercise induces CREB activation [17,18], relating that to a physiological response to the increased oxidative metabolism [19] and mitochondrial biogenesis [20].

Molecular hydrogen (H_2) present in nature and in chemicals products has been revealed as a promising new therapeutic gaseous molecule [21]. H_2 treatment ameliorates clinical aspects of carcinogenic diseases [22], cardiovascular diseases [23,24], neuromuscular and neurodegenerative diseases [25,26], diabetes and metabolic syndrome [27,28], acute injuries to soft tissue and skin disease [29], renal disorders [30,31], and inflammatory diseases [32,33]. Initially, H_2 was thought to be an inert gas. However, recent evidence indicates that H_2 is able to neutralize the hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) within the cells, promoting cytoprotective effects against oxidative stress [34]. Studies in rodents have shown that H_2 inhalation provides beneficial effects damage in organs such as the heart [35], lungs [36], liver [37], ischemia-reperfusion in the brain [38] and in a model of sepsis [39]. Moreover, H_2 has been shown to promote anti-allergic, anti-apoptotic, and anti-inflammatory effects, emerging as a cellular protector [40]. More specifically, H_2 has been reported to have an antifatigue effect in mice [41] and humans [42] and to suppress exercise-induced oxidative stress in Thoroughbred horses when intravenously infused [43,44]. Despite this intense production of knowledge related to H_2 actions, there are no reports in the literature about the possible effect of H_2 inhalation on physical exercise-induced inflammation and ROS production.

Therefore, we tested the hypothesis that H_2 decreases acute physical exercise-induced inflammatory and oxidative stress status in sedentary rats. To achieve this goal, rats ran on a closed treadmill inhaling an H_2-containing mixture. Then, we evaluated plasma levels of TNF-α, IL-1β, IL-6, SOD, TBARS, and nitrite/nitrate (NOx - that indicates the endogenous production of NO) as well as assessed GSK3α/β and CREB phosphorylation status in the skeletal muscle.

2. Materials and methods

2.1. Animals

A total of sixty male Wistar rats weighing 200–220 g were used. The rats had free access to water and food, and were maintained in cages (with 4 rats each) with a metallic grid lid and the floor covered with wood chip bedding material and were housed in a temperature-controlled chamber at 24 ± 1 °C (model: ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil), with a 12:12-h light:dark cycle (lights on at 6:00 a.m.). The animals were randomly divided in eight groups and euthanized immediately (0 h) or 3 h after the acute physical exercise bout: (i) sedentary inhaling the control gas (Sed Air 0 h or 3 h); (ii) sedentary inhaling the H_2 gas (Sed H_2 0 h or 3 h); (iii) exercise inhaling the control gas (Ex Air 0 h or 3 h); and (iv) exercise inhaling the H_2 gas (Ex H_2 0 h or 3 h). Experiments started between 08:00 and 12:00 a.m. to prevent effects of circadian variation. Animal care was carried out in compliance with the guidelines set by the Guide for the Care and Use of Laboratory Animals of the National Council for the Control of Animal Experimentation (CONCEA). Experimental protocols were approved by the Local Animal Ethical Committee of the Dental School of Ribeirao Preto, University of Sao Paulo (2017.1.893.58.5).

2.2. Maximal exercise test

The maximal exercise test was performed with 3 m/min increments every 3 min, until the rats were unable to run further. The maximal exercise test was utilized to determine the maximum running velocity (Vmax). This protocol is based on pilot experiments in our laboratory that produced the most repeatable and consistent results, as well as on previous studies [45,46].

2.3. Experimental design

Rats were adapted on a motor-driven treadmill enclosed (AVS model) for 3 days to 10 min/day and the speeds/day was 12, 15, 18 m/min (0° slope). On the fourth day, the maximal exercise test was performed to determine the Vmax. Then, the rats rested for 1 day and on the sixth day the experiment took place (Fig. 1).

2.4. H_2 measurements

Similarly to previous studies [47,48], H_2 gas (2% H_2, 21% O_2, balanced with N_2) and control gas (0% H_2, 21% O_2, balanced with N_2) were purchased from White Martins Gases Industriais Ltda (Sertãozinho, SP, Brazil). Additionally, a gas analyzer (DG-700, Instrutherm, São Paulo, Brazil) was used to continuously monitor the H_2 concentration in the outflow hose of the treadmill chamber.

2.5. Experimental protocol

The H_2 concentration of 2% was chosen on the basis of previous studies [25,34,35,47,49] and because in pilot experiments, this

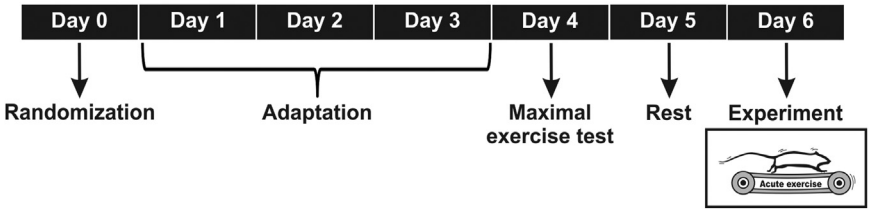

Fig. 1. Illustration of the overall experimental procedure: Randomization (day 0): rats were randomized before adaptation. Adaptation: rats were adapted on a closed motor-driven treadmill for 3 days to 10 min/day and the speeds/day was 12, 15, 18 m/min (0° slope). Maximal exercise test (fourth day): the maximal exercise test was performed to determine the maximum running velocity (Vmax). Rest (fifth day): the rats rested. Experiment (sixth day): Each rat was enclosed in the treadmill for 30 min acclimatizing and inhaling either the H_2 gas (2% H_2, 21% O_2, balanced with N_2) or the control gas (0% H_2, 21% O_2, balanced with N_2). Thereafter, they ran for 30 min at the speed corresponding to 80% of Vmax inhaling the H_2 gas or the control gas. Blood samples (plasma) and the soleus muscles from hind paws were collected immediately or 3 h after the acute physical exercise.

concentration produced the most consistent and repeatable responses. Each rat was kept inside of the enclosed motor-driven treadmill for 30 min acclimatizing and inhaling either the H_2 gas (2% H_2, 21% O_2, balanced with N_2) or the control gas (0% H_2, 21% O_2, balanced with N_2). Thereafter, they ran for 30 min at the speed corresponding to 80% of Vmax inhaling the H_2 gas or the control gas. The inflow of the H_2 gas and the control gas was 2.4 L/min, controlled by a flowmeter. Sedentary rats inhaled the H_2 gas or control gas during the same time of the exercised groups. Samples of blood and the soleus muscles from hind paws were collected immediately or 3 h after the acute physical exercise.

2.6. Plasma and muscle collection

Sedentary and trained rats were euthanized immediately or 3 h after the acute physical exercise bout. Blood was collected in heparin-coated tubes, centrifuged (3.500 rpm, 20 min, 4 °C) and plasma and stored at −70 °C. The soleus muscles were excised, promptly frozen by submersion in dry ice-cold isopentane, and stored at −70 °C.

2.7. Measurement of cytokines plasma levels

Cytokines (TNF-α, IL-1β and IL-6) plasma levels were determined using specific enzyme-linked immunosorbent assay (ELISA) kits for each cytokine (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. The results were expressed in pg/mL.

2.8. Measurement of SOD activity plasma levels

SOD activity plasma levels were measured spectrophotometrically using the specific assay kit (SOD Assay Kit, Cayman, code 706002) according to the manufacturer's instructions. The results were expressed in U/mL.

2.9. Measurement of TBARS plasma levels

TBARS plasma levels were performed using the colorimetric method, following the kit recommendations (TBARS Assay Kit, Cayman, code 10009055). TBARS concentration was calculated by standard curve of bis-malonaldehyde and the results were expressed as nmol/mL.

2.10. Measurement of NOx plasma levels

Plasma samples (50 µl) were deproteinized by precipitation using 100 µl of absolute ethanol maintained at 4 °C, followed by stirring and remained for 30 min in a freezer (-20 °C). Then, it was centrifuged (10.000 rpm, 10 min, 25 °C) for further measurement. The NO/ozone chemiluminescence technique was used by the Sievers® Nitric Oxide Analyzer 280 analyzer (GE Analytical Instruments, Boulder, CO, USA). The volume of 5 µl of sample was injected into the analyzer reaction chamber containing the reducing agent 0.8% vanadium chloride in 1 N HCl at 95 °C. The results were normalized by the protein concentration of each sample and expressed as µM/mL.

2.11. Western blotting

Samples of the soleus muscles from hind paws were homogenized in Tris-HCl buffer (50 mM, pH 7.4, 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% sodium deoxycholate, 1% SDS and 1% Triton X-100), homogenized with a cocktail of proteases and phosphatases inhibitors (1:100, Cell Signaling, Danvers, MA, USA). Tissue homogenates were centrifuged at 14.000 rpm for 20 min at 4 °C and the supernatant was collected for analysis. Total protein concentration in supernatants was determined by a Bradford-based assay (#5000205, Bio-Rad Laboratories, USA Laboratories, USA) [50] and used for normalization. Aliquots containing 30 µg of protein were mixed in an equal volume of sample buffer (125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 20% glycerol and 0.02% bromophenol blue, pH 6.8) and boiled at 70 ± 3 °C for 10 min. Proteins were separated by electrophoresis on 10% SDS-PAGE Tris-glycine gels (Bio-Rad Laboratories, USA) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, USA). PVDF membranes were blocked in a bovine serum albumin solution [3% BSA in Tris buffered saline with 0.1% Tween (TBS-T)] for 1 h at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies [anti-phospho-GSK3α/β or anti-phospho CREB, (both 1:1000, Cell Signaling, Danvers, MA, USA)] diluted in TBS-T solution with 5% BSA. In the next day, membranes were washed 3 times per 5 min each with TBS-T and incubated with HRP-conjugated secondary antibodies (rabbit anti-IgG or mouse anti-IgG antibodies, both 1:3000, Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. Bands were visualized using the ChemiDoc system (Bio-Rad Laboratories, USA) The recovery process of the membranes for re-incubation was performed as previously described [80]. Briefly, the membranes were covered with 100% ethanol for 1 min and then submerged in 29% hydrogen peroxide for 15 min at 37 °C, avoiding light. Then, the membranes were blocked for 18 h and incubated overnight with the primary antibody. The following procedures were performed as described above. The bands were quantified using the ImageLab software (Bio-Rad Laboratories, USA) with simultaneous normalization using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping control by using the anti-GAPDH primary antibody (1:3000, Cell Signaling, Danvers, MA, USA). The protein/GAPDH ratio was quantified as a percentage of the control group (Sed Air 0 h), which was considered to be 100%.

2.12. Statistical analysis

Data are expressed as means ± standard error of mean (SEM). One-way ANOVA, followed by Bonferroni´s post hoc test, was used to determine the statistical differences among the groups. The level of significance was set at $p < 0.05$.

3. Results

3.1. Effect of physical exercise combined or not with H_2 on cytokines plasma levels

We evaluated plasma levels of cytokines (TNF-α, IL-1β and IL-6) to verify the potential anti-inflammatory effect of H_2 during physical exercise. Physical exercise increased plasma TNF-α level when compared to sedentary groups immediately. H_2 inhalation significantly blunted ($p < 0.05$) the exercise-induced TNF-α production immediately after a bout of exercise compared to the respective control group (Fig. 2A). IL-1β levels were non-detectable in all experimental groups (Fig. 2B). Physical exercise increased significantly ($p < 0.05$) plasma IL-6 concentration compared to sedentary groups. H_2 inhalation significantly blunted ($p < 0.05$) exercise-induced IL-6 surge immediately compared to the respective control group (Fig. 2C).

3.2. Effect of physical exercise combined or not with H_2 on SOD plasma levels

Antioxidant defense status was evaluated by plasma SOD activity measurements. Physical exercise increased plasma SOD activity immediately after the section and the H_2 inhalation did not affect this increased exercise-induced SOD activity. In control rats, kept under 0% H_2, euthanized 3 h after exercise SOD activity was observed to be back to its nadir levels whereas H_2 inhalation caused a maintained higher SOD activity levels (Fig. 3).

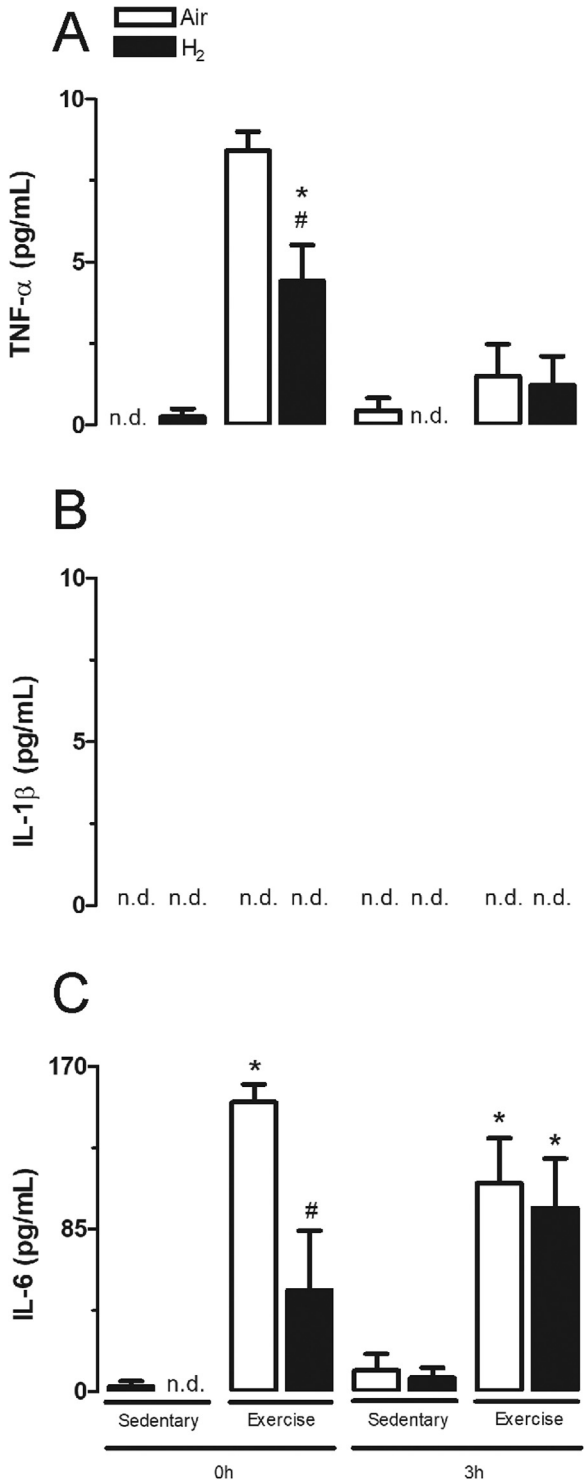

Fig. 2. Plasma levels of TNF-α (A) IL-1β (B) and IL-6 (C) of rats euthanized immediately (0 h) or 3 h after acute exercise inhaling or not H_2. (n = 6–8/group). $^{\#}p < 0.05$ vs. Ex Air 0 h, $^{*}p < 0.05$ vs. the respective control group. n.d., Nondetectable levels.

3.3. Effect of physical exercise combined or not with H_2 on the lipid peroxidation

Systemic oxidative stress status was evaluated by plasma TBARS measurements. Immediately after the exercise we did not observe any change in TBARS levels. Three hours after physical exercise, an increased TBARS levels was observed which was blunted by H_2 inhalation

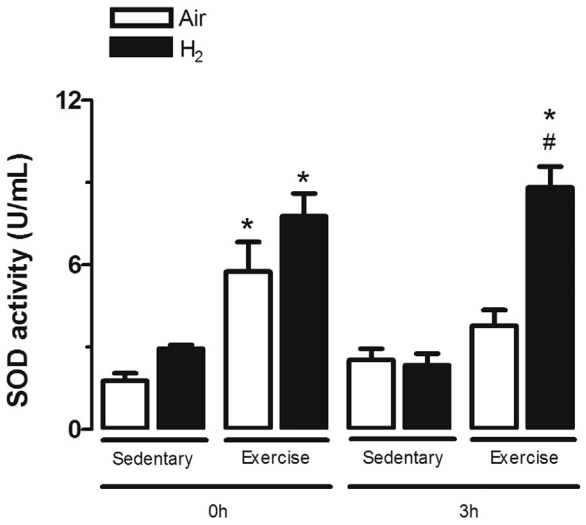

Fig. 3. Plasma levels of superoxide dismutase (SOD) of rats euthanized immediately (0 h) or 3 h after acute physical exercise inhaling or not H_2. (n = 6–8/group). $^{\#}p < 0.05$ vs. Ex Air 3 h, $^{*}p < 0.05$ vs. the respective control group.

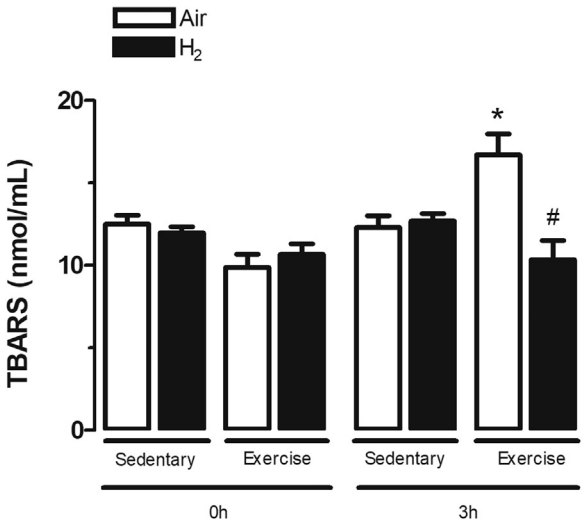

Fig. 4. Plasma levels of thiobarbituric acid reactive substances (TBARS) from rats euthanized immediately (0 h) or 3 h after acute physical exercise inhaling or not H_2. (n = 6–8/group). $^{\#}p < 0.05$ vs. Ex Air 3 h, $^{*}p < 0.05$ vs. the other groups.

(Fig. 4).

3.4. Effect of physical exercise combined or not with H_2 on plasma NOx levels

Besides, SOD activity, the antioxidant defense status was also evaluated assessing plasma NOx levels. Exercise caused an increased NOx production that was not affected by H_2, both immediately and 3 h post-exercise (Fig. 5).

3.5. Effect of physical exercise combined or not with H_2 on muscle GSK3α/β and CREB phosphorylation

To assess intracellular oxidative stress and metabolism we measured the relative phosphorylation of GSK3α/β and CREB in soleus muscle of sedentary or exercised rats inhaling or not H_2. These two proteins were detected in soleus muscles in all experimental groups (Fig. 6A). No statistical difference between groups was found in the profile of

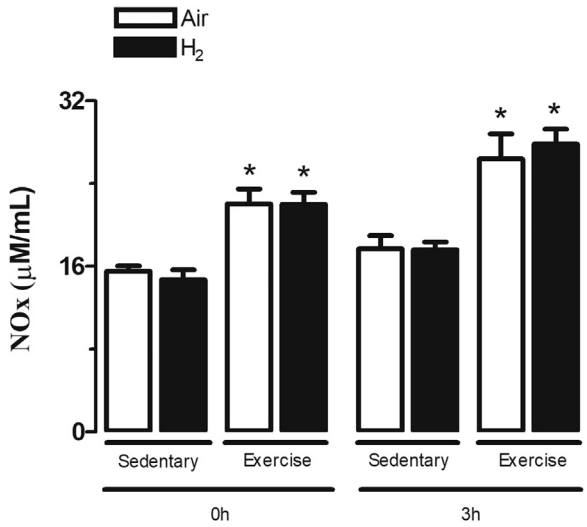

Fig. 5. Plasma levels of nitrite/nitrate (NOx) of rats euthanized immediately (0 h) or 3 h after acute physical exercise inhaling or not H_2. (n = 6–8/group). *p < 0.05 vs. the sedentary groups.

expression of phospho-GSK3α/β (Fig. 6B). Differently, physical exercise significantly increased ($p < 0.05$) CREB phosphorylation 3 h after exercise compared to the other groups. Interestingly, H_2 inhalation reduced CREB phosphorylation after physical exercise (Fig. 6C).

4. Discussion

This is the first study to report that inhalation of H_2 reduces inflammatory and positively modulates oxidative stress caused by a physiological stimulus, i.e., an acute physical exercise bout. Since muscles are considered a secretory organ during and after physical exercise [51], it is plausible to relate systemic markers as a result of muscular activation. The inflammatory process that takes place in worked muscles is beneficial when exercise is regular or detrimental when exercise is relatively intense and/or performed by unaccustomed individuals [4]. In this study, untrained rats ran at 80% of Vmax for 30 min, characterizing an intense exercise [52] to untrained subjects. Our data clearly show that H_2 exerts an anti-inflammatory effect by blunting exercise-induced surges of plasma levels of TNF-α and IL-6 (Fig. 2) in rats euthanized immediately after exercise, but not 3 h after the session. These results are consistent with the notion that the effect of H_2 is relatively more effective acutely than in the long term. Despite the existence of several studies assessing the exercise-induced increases in plasma cytokines levels [1] this issue is far from completely

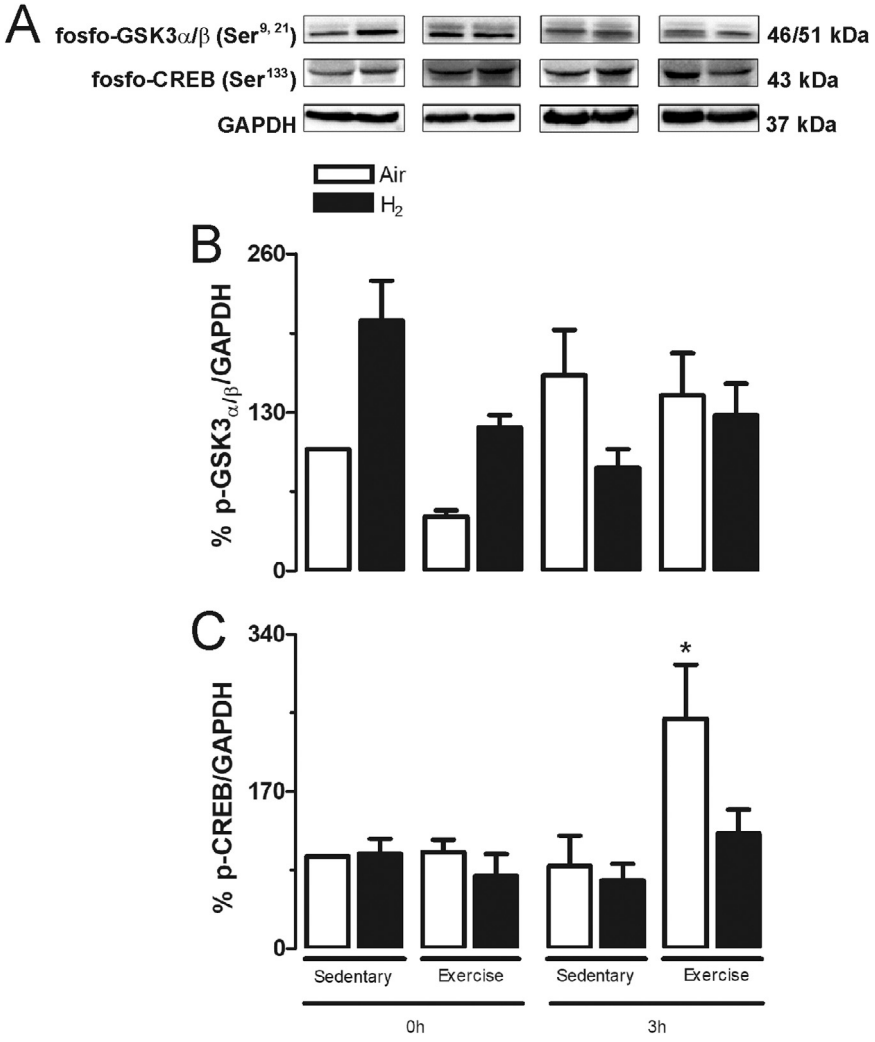

Fig. 6. Panel A shows representative bands of each experimental group. Relative expression of (B) phospho-GSK3α/β ($Ser^{9,21}$) and (C) phospho-CREB (Ser^{133}) expressed as percentage of the fraction between the protein and GAPDH (control) in the soleus muscle of rats euthanized immediately (0 h) or 3 h after acute physical exercise inhaling or not H_2. (n = 4–10/group). * p < 0.05 vs. the other groups.

understood [53].

In the present study, the exercise-induced surges in plasma TNF-α, and IL-6 were inhibited by H_2, whereas no change in plasma IL1-β levels were observed. Previous studies agree with the present results demonstrating reduced surges of pro-inflammatory cytokines in the plasma of rats submitted to other stressors such as during immune challenge [54]. After exercise, IL-1β and TNF-α expression have been reported to be increased in skeletal muscle, but circulating levels of these cytokines have small changes or are not detectable, whereas IL-6 levels are consistently reported to be increased in both muscle and plasma [2]. In our study, IL-6 was found to be increased in animals euthanized immediately and 3 h after exercise, corroborating the literature.

Many studies have demonstrated the beneficial effects of H_2 on diseases in humans and experimental animal models [21–39], but the mechanisms responsible for these benefits are not fully understood. Our data demonstrate that H_2 may also modulate the inflammatory response caused by a physiological stimulus, i.e., physical exercise, decreasing plasma levels of TNF-α and IL-6. To our knowledge, there is no study in the literature that integrates the effect of H_2 on inflammatory and oxidant responses produced by physical exercise, although there are different experimental models relating the anti-inflammatory effect of H_2. Rats in a model of hepatic ischemia-reperfusion injury showed reduced plasma levels of TNF-α, IL-1β and IL-6 after inhalation of 2% H_2 for 1 h [55] and mice that inhaled H_2 showed reduced plasma levels of TNF-α and lipid peroxidation in a chronic liver inflammation model [56]. In a more mechanist view, Iuchi et al., [57] have recently proposed that H_2 may exercise its modulatory effect on inflammation acting on the free radical chain oxidation that generates oxidized lipid mediators before calcium signaling is activated. This oxidized phospholipids production would be reduced by H_2 because of a modulation of the free radical chain reactions. The oxidized phospholipids would lead to a reduced calcium signaling, eventually causing inhibition of TNF-α and COXII expressions.

In the present study, we observed that anti-inflammatory effects of H_2 occur earlier than the inhibitory effects of the gas on SOD activity and TBARS production (Figs. 2 and 3). This observation may indicate that: (i) the effects of H_2 on SOD activity and TBARS production are indirectly mediated by the reduced cytokines production; or (ii) the two parameters are affected by H_2 directly but with different timing. Further studies are needed to make this issue clear.

Exercise induces muscle damage that activates leukocytes, mainly neutrophils and monocytes/macrophages, which are cytokine producing cells, as are the muscles fibers themselves [58]. Cytokines are key factors inducing the systemic inflammatory response after acute exercise [2,59,60]. The type of exercise, intensity and duration affect the magnitude of cytokines release [59,61]. In this study, acute exercise induced TNF-α and IL-6 surges that were attenuated by H_2 (Fig. 2). Many studies have shown that H_2 decreases plasma levels of pro-inflammatory cytokines, exerting an anti-inflammatory effect [55,56,62]. However, the exact mechanism by each H_2 activates this anti-inflammatory response is not fully understood. It is known that H_2 may act in at least two different ways to decrease inflammation: (i) H_2 can act directly on leukocytes as previously reported in studies that have documented a powerful anti-inflammatory effect of H_2 in lymphocytes [63] and macrophages [48,64,65]; and (ii) H_2 may down-modulate ROS production [34]. However, it is worth to mention that this is a fairly complex system in which both inflammation and oxidation processes may be reciprocally related, since it has been reported that ROS plays a positive role on cytokines production by up-regulating NF-kB signaling pathway [66]. Therefore, we suggest that the decreased TNF-α and IL-6 plasma surges of rats that inhaled H_2 (Fig. 2) may result from a direct action of H_2 on leukocytes, but not due to an indirect action of the gas on ROS production, since no effect of H_2 was observed on exercise-induced increased SOD activity immediately after the bout (Fig. 3).

Physiological levels of ROS are essential for cell signaling. However, ROS overproduction, for instance induced by physical exercise, can cause tissue damage and oxidative stress [5]. SOD is an important enzyme that is involved in the regulation of cellular antioxidant defenses [67]. In this study, rats had increased SOD plasma levels after running for 30 min at 80% of Vmax. Our study corroborates other studies showing that SOD activity increases after a single bout of acute exercise [7,68,69]. Interestingly, we observed a tendency of an early (immediately after exercise) increase in SOD activity in the rats that performed the acute exercise inhaling H_2. This tendency was confirmed when we measured SOD activity 3 h after exercise, and a significant increase was observed in rats that run under 2% H_2, further indicating an anti-oxidative role of the gas (Fig. 3). There is no evidence in the literature about the interaction of physical exercise, SOD activity and H_2. In a model of allergic airway inflammation, mice that inhaled H_2 for 7 days had increased SOD activity in lung tissue [70] and mice with rheumatoid arthritis treated with H_2 showed also increased SOD activity [71]. Moreover, SOD activity was increased in rats that drank H_2-rich water on the intestinal ischemia/reperfusion injury [72]. Therefore, our findings corroborate previous studies using other experimental models showing the powerful anti-oxidative effect of H_2.

Intense physical exercise causes an increase in the oxygen consumption and consequently the amount of free radicals, exceeding the production of antioxidants, causing the oxidative stress [73]. Oxidative stress induced by intense physical exercise can cause damage to several cellular components, such as DNA, proteins and membrane lipids, which can be detected by increased lipid peroxidation, observed in both humans and rats [74]. In the present study, lipid peroxidation was evaluated by TBARS measurements (Fig. 4). Plasma TBARS levels were increased in control exercised rats euthanized 3 h after exercise, which corroborates with the literature [74]. In rats that ran under 2% H_2, exercise-induced increases in TBARS levels were found to be attenuated 3 h after the exercise bout, further indicating an antioxidant effect of the gas. Once more, there are no previous studies that relate the effect of H_2 and exercise in response to the lipid peroxidation markers. However, it is interesting to note that humans with a potential metabolic syndrome who ingested water enriched with H_2 for 8 weeks present a decrease in TBARS levels [75]. In fact, H_2 was revealed as a new antioxidant agent, but its putative molecular targets are still being identified. For instance, it has been suggested that H_2 modulates Ca^{2+} signal transduction and gene expression modifying the production of phospholipid species [57].

Physical exercise caused an increase in NOx production (Fig. 5), as previously reported [76,77]. It is believed that this increase is due to the greater shear force observed during the exercise [78]. Exercise caused a higher NOx plasma levels compared to sedentary rats (Fig. 4), which corroborates with the literature for both humans and rats [76,77]. Our data indicate that H_2 is not able to alter NOx plasma levels neither in sedentary rats nor the exercise-induced increase in NOx production.

Several studies show that H_2 can modulate intracellular cascades, producing favorable effects modulating oxidative stress and cellular metabolism [79]. Among the proteins that modulate these pathways within the cells, GSK3α/β plays an important role in reducing mitochondrial ROS production [14,80] and CREB that acts in response to increased oxidative metabolism [19] besides being recruited during physical exercise, producing adaptive muscle responses [17]. In the present study, GSK3α/β phosphorylation was not statistically altered by neither exercise nor by H_2 (Fig. 6B). Interestingly, we observed a tendency of increase in GSK3α/β phosphorylation in the H_2-treated groups that may indicate changes in cell metabolism or in the oxidative status of the muscle. The increase of GSK3 activity in response to physical exercise is time and intensity dependent [81]. GSK3 has been described as an regulator of glycogen synthase metabolism [82,83], which plays a meaningful role in glycogen synthesis [84]. GSK3 can also induce CREB phosphorylation [16]. Our results suggest that, in our experimental protocol, CREB was not stimulated by GSK3. However, exercise

increased CREB phosphorylation which was attenuated by H_2 3 h after exercise (Fig. 6C). This phenotype appears to be also present in mice and humans, in which different experimental protocols of exercise is reported to induce the activation of CREB in a temporary way [17,18].

5. Conclusion

This study provides evidence that H_2 reduces inflammation, oxidative stress and cellular stress induced by acute physical exercise, based on the measurement of plasma concentration of TNF-α, IL-6, TBARS, SOD activity, and CREB phosphorylation in the skeletal muscle. These findings shed light on the effect of the gas that has been reported to have no side effects and, therefore, may be safely used to modulate physical exercise-induced inflammation and oxidative stress.

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Conflict of interest

The authors declare no conflict of interest.

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