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Unsaturated fatty acids from flaxseed oil and exercise modulate GPR120 but not GPR40 in the liver of obese mice: a new anti-inflammatory approach☆

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Abstract

GPR120 and GPR40 were recently reported as omega-3 (ω3) receptors with anti-inflammatory properties. Physical exercise could increase the expression of these receptors in the liver, improving hepatic metabolism in obesity and type 2 diabetes. Our aim was to investigate GPR120/40 in the liver of lean and obese mice after acute or chronic physical exercise, with or without the supplementation of ω3 rich flaxseed oil (FS), as well as assess the impact of exercise and FS on insulin signaling and inflammation. Mice were fed a high-fat diet (HF) for 4 weeks to induce obesity and subsequently subjected to exercise with or without FS, or FS alone. Insulin signaling, inflammatory markers and GPR120/40 and related cascades were measured. Chronic, but not acute, exercise and FS increased GPR120, but not GPR40, activating β-arrestin-2 and decreasing the inflammatory response, as well as reducing fat depots in liver and adipose tissue. Exercise or a source of ω3 led to a higher tolerance to fatigue and an increased running distance and speed. The combination of physical exercise and ω3 food sources could provide a new strategy against obesity through the modulation of hepatic GPR120 and an increase in exercise performance.

Keywords: Obesity; GPR120; Exercise; Unsaturated fatty acids; Omega-3

1. Introduction

Obesity is at record levels [1], and therefore, any advancement in understanding the relationship between physical activity and balanced nutrition beyond simple energy expenditure or calorie restriction could provide huge benefits in this area. Studies have shown, for example, the ability of nutrients, such as omega-3 (ω3), and physical exercise to reduce inflammation and endoplasmic reticulum stress in hypothalamus, partially restoring food intake control [2,3] and modulating the peripheral inflammatory state [4], which contributes to the improvement of insulin resistance and glucose clearance.

With ω3 able to modulate inflammation and effect obesity-related outcomes, it is of interested that two G-protein coupled receptors

Abbreviations: Akt, protein kinase B; ALA, alpha linolenic acid; APPL1, adaptor protein, phosphotyrosine interaction, pleckstrin homology domain and leucine zipper containing 1; βarrestin2, beta arrestin 2; CT, control group; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Exe, exercise; FS, flaxseed; GPCR, G protein coupled receptor; GPR120/40, G protein coupled receptor 120 or 40; GSK3, glucogen synthase kinase 3; GTT, glucose tolerant test; HF, high fat; IκK, inhibitor kappa kinase; IκBα, NF-κB inhibitor-alpha; IL, interleukin; I.P, intraperitoneal; IR, insulin receptor; ITT, insulin tolerant test; JNK, c-Jun N-terminal kinase; kDa, kilodalton; K_{ITT}, insulin tolerant test constant decay; MUFA, monounsaturated fatty acid; NAFLD, nonalcoholic fat liver disease; NLRP3, nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPARγ, peroxisome proliferator-activated receptor gamma; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAB1/2, transforming growth factor beta-activated kinase1/2 binding protein; TAK1, transforming growth factor activated kinase 1; TLR2/4, toll like receptor2/4; TNFα, tumoral necrosis factor alpha; TNF-R, tumoral necrosis factor-receptor; ω3, omega-3

☆ Conflict of interests: no conflicts of Interest.

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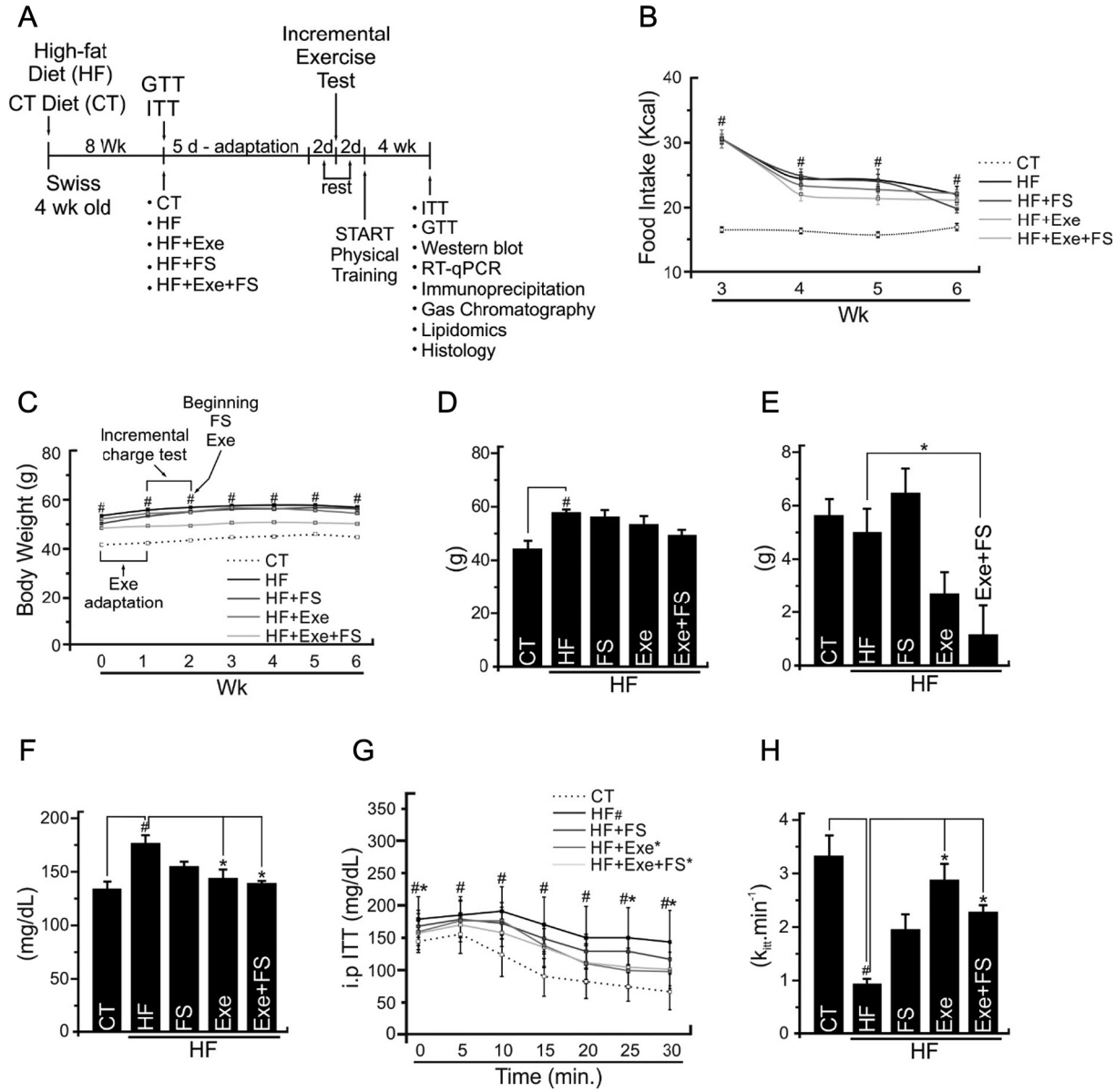


Fig. 1. Experimental protocol design; chronic exercise and metabolic characterization. (A) Four-week-old male Swiss mice were randomly assigned to a regular chow (CT) or high-fat diet (HF) over 2 months in total. Four weeks after beginning the HF diet, mice were tested for insulin resistance and sensitivity and glucose tolerance and then randomly distributed into four groups: HF (HF), HF plus exercise (HF + Exe), HF plus FS oil treatment (100 μl) (HF + FS) and HF plus exercise and FS oil treatment (100 μl) (HF + Exe + FS). After 5 days of exercise adaptation and 2 days of rest, mice were submitted to an incremental exercise test to determine the maximum potency of exercise and subsequently began exercise training for 4 weeks. On the last day of experimental period, the animals were killed and the liver was removed for analysis. After the 4 weeks, food intake (B), body mass variation (C and D), body weight evaluation (E), fasting glucose (F) and insulin sensitivity (G and H) were evaluated. CT (n=6), HF (n=7), HF + FS (n=8), HF + Exe (n=7) and HF + Exe + FS (n=8). Values are expressed as means ± S.D. $^{\#}P<.05$ *vs.* CT group by Student's *t* test. $^{*}P<.05$ *vs.* HF (Tukey's test). CT, regular chow; HF, high-fat diet; FS, flaxseed oil; Exe, exercise.

(GPCR), GPR120 and GPR40, were recently deorphanized and shown to recognize ω3 acids (docosahexaenoic, C22:6; eicosapentaenoic, C20:4; and α-linolenic, C18:3), as well as omega-9 (oleic acid, C18:1) [5,6]. Following GPR120/40 activation, βarrestin2 binds to GPR120/40 and internalizes the receptor and its agonist. Concomitantly, βarrestin2 recruits the transforming growth factor beta-activated kinase1/2 binding protein (TAB1/2) from proinflammatory pathways, including Toll-like receptor (TLR) 2/4 and tumor necrosis factor alpha (TNF-α) pathways, disassembling their cascades [4,5,7,8]. In addition, βarrestin2 has been shown to disrupt the structure of the inflammasome by binding to the nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) protein, following ω3 fatty acid induced GPR120 activation, reducing the inflammation [9].

The mechanisms by which exercise has been shown to be beneficial in metabolic diseases include the decrease of TLR4 signaling improving insulin sensitivity *via* interleukin (IL)-6 and peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α mediated pathways [10–13]. Furthermore, exercise has been shown to increase APPL1 (adaptor protein, phosphotyrosine interaction, pleckstrin homology domain and leucine zipper containing 1), restoring insulin receptor (IR) and Akt signaling [14], as well as increasing the production of anti-inflammatory IL10. Exercise is also the accepted treatment in nonalcoholic fatty liver disease (NAFLD) [15].

Given the potential of both exercise and ω3 in improving health state in obesity and insulin resistance/type 2 diabetes mellitus, we hypothesized that acute and chronic exercise could increase insulin sensitivity and exercise performance, decrease inflammation and

importantly increase the expression of GPR120 and GPR40 in the liver and that these effects could be enhanced by ω3 supplementation (FS oil). Herein, GPR120 but not GPR40 seems to be positively modulated by exercise and FS, decreasing the hepatic inflammation induced by increased fat consumption.

2. Methods

2.1. Experimental animals

After Ethical Committee acceptance (#3512-1), 4-week-old, male, Swiss Albinus mice were housed in individual cages at 21°C±2°C with a 12-h light/dark cycle. The Swiss mouse readily develops obesity and associated comorbidities on an HF diet alone. At the end of experiment, the animals were anesthetized and euthanized with high levels of anesthesia (see section 2.7, method).

2.2. Experimental design and diet

The acute or chronic exercise protocols in Ferreira et al. (2007) [16] were followed. An acute exercise program (Fig. S1A) was initially carried out to test the best moment of GPR120 expression in the liver. Animals (n=5/group) following the acute program and sedentary controls were fed a standard rodent chow, and liver fragments were removed at 0 h, 8 h, 16 h, 24 h or 48 h postexercise. The liver was chosen based on this organ's crucial role in energetic metabolism.

In the primary study, we aimed to understand whether chronic exercise could increase the GPR120 expression and protein content in the liver from obese mice (Fig. 1A). Then, during 8 weeks, mice were fed either the standard rodent chow (CT group), purchased from Nuvilab, or a high-fat (HF) alternative (HF group), prepared in accordance to American Institute of Nutrition Guidelines (AIN-93G) [17]. In the HF diet, 31% of the corn starch was replaced by lard [7] as described in Table 1. Those receiving the standard chow remained sedentary (CT). Those on the HF diet were subdivided into four groups: (1) sedentary mice (HF), (2) chronic exercise (HF + Exe), (3) sedentary mice with flaxseed oil (HF + FS) and (4) chronic exercise with FS oil (HF + Exe + FS). The chronic exercise program ran over a 4-week period, and all mice received either the FS (Supplemental Table 1) or saline daily *via* gavage at a dose of 100 μl per mouse. FS was chosen because of its high concentration of ω3 [52.3% α-linolenic acid (C18:3)], which is naturally present in foods, and because of its low cost and low levels of adulteration compared to fish oil. Saline was considered the most appropriate control due to the potential of other oils, such as corn or sunflower oil, to induce a proinflammatory status due to their high linoleic fatty acid content [18]. At the end of experimental period, liver fragments were removed at 24 h postexercise (Fig. 1A).

To test the insulin signaling, mice from each group were aleatory selected to receive an injection of insulin (100 μl 10^{-6} mol/L) or saline (100 μl) through the portal vein. After 30 s, fragments of hepatic tissue were removed and immediately homogenized in extraction buffers (see section 2.7, method).

2.3. Physical exercise protocols (acute and chronic)

Both exercise protocols (acute and chronic) consisted of running on a motor treadmill at a 60% intensity of the peak workload. The acute exercise protocol consisted of a single bout of running on a motor treadmill for 60 min (Fig. S1). The chronic exercise program consisted of 5 days a week for 4 weeks; however, the length of training started at 15 min per day and was gradually increased by 15 min over each subsequent week (i.e., by week 4, mice were carrying out 60 min of exercise per day) (Fig. 1A).

2.4. Reagents and antibodies

The reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA, USA). Human recombinant insulin (Humulin R) was from Lilly (Indianapolis, IN, USA). Anti-Akt (sc-8312) rabbit polyclonal; anti-phospho [Thr 183/185] c-Jun N terminal kinase (sc-6254) mouse monoclonal; anti-IL10 (sc-1783) goat polyclonal; anti-α-tubulin (sc-398,103) mouse monoclonal; anti-GPR120 (sc-48,203) goat polyclonal; anti-GPR40 (sc-32,905) rabbit polyclonal; anti-βarrestin2 (sc-13,140) mouse monoclonal; anti-glyceraldehyde 3-phosphate dehydrogenase (sc-25,778) rabbit polyclonal and anti-inhibitor kinase kappa (sc-34,673) rabbit polyclonal were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The adopted dilution was 1:1000 for each Santa Cruz antibody. Anti-phospho-glycogen-synthase-kinase-3β [Ser9] (#5558) rabbit polyclonal, anti-glycogen-synthase-kinase-3β (sc9166) rabbit polyclonal, anti-phospho-TAK1 [Ser412] (#9339) rabbit polyclonal, anti-phospho-Akt [Ser 473] (#4051) mouse monoclonal, anti-phospho-NF-κB Inhibitor alpha (IKBα) [Ser32/36] (#9246) mouse monoclonal and anti-phospho inhibitor kinase kappa (#2697) mouse monoclonal were from Cell Signaling (Danvers, MA, USA). The adopted dilution was 1:2000 for each Cell Signaling antibody. Anti-IL1β (503502 mouse-rat) and anti-TNFα (506101, mouse-rat) were from BioLegend (San Diego, CA, USA). The adopted dilution was 1:2000 for each BioLegend antibody.

2.5. Intraperitoneal insulin tolerance test

The insulin tolerance test (ITT) was carried out to guarantee the insulin resistance induced by HF diet and to evaluate the effectiveness of the treatments. Then, after 8 h of fasting, insulin (1.5 U/kg body weight^{-1}) was injected i.p., and blood samples were collected from the tail vein at 0 min and every subsequent 5 min for 30 min for serum glucose determination. The constant for the rate of serum glucose decay was calculated using the formula 0.693/biological half-life ($t_{1/2}$). The plasma glucose $t_{1/2}$ was calculated from the slope of last square analysis of the plasma glucose concentration during the linear phase of decline [19]. Glucose levels were determined using Accutrend Plus equipment (Roche, Switzerland).

2.6. Intraperitoneal glucose tolerance test

With the same proposal of ITT, the glucose tolerance test (GTT) was carried out. After 8 h of fasting, a blood sample was collected from the

Table 1
Diet based on AIN-93G (American Institute of Nutrition, 1993) [17].

HF diet components			
Ingredients	(g/kg^{-1})	kcal/kg^{-1}	% Fatty acids
Corn starch	115.5 g	462	
Casein	200 g	800	
Sucrose	100 g	400	
Dextrinated starch	132 g	528	
Soybean oil	40 g	360	
Lard	312 g	2808	
Cellulose	50 g	-	
Mineral mix	35 g	-	
Vitamin mix	10 g	-	
L-Cysteine	3 g	-	
Choline	2.5 g	-	
SFA	-	-	37.48
MUFA	-	-	42.56
PUFA	-	-	19.96
*ALA (C18:3)	-	-	1.29[a]
Total	1000 g	5358 Kcal	100%

HF, high-fat [7]; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ALA, alpha linolenic fatty acid.
* ALA is a nutrient included in the PUFA.

tail vein (time 0) prior to the i.p. administration of a 25% glucose solution (2.0 g/kg body weight). Further blood samples were then collected every 30 min for 120 min to determine blood glucose concentrations. Results are presented as the area under the glucose curves.

2.7. Immunobloting

After an i.p. injection of anesthesia [ketamine (50 mg/kg body weight^{-1}) and xylazine (20 mg/kg body weight^{-1})], corneal reflexes were confirmed as absent, and the abdominal cavities were opened.

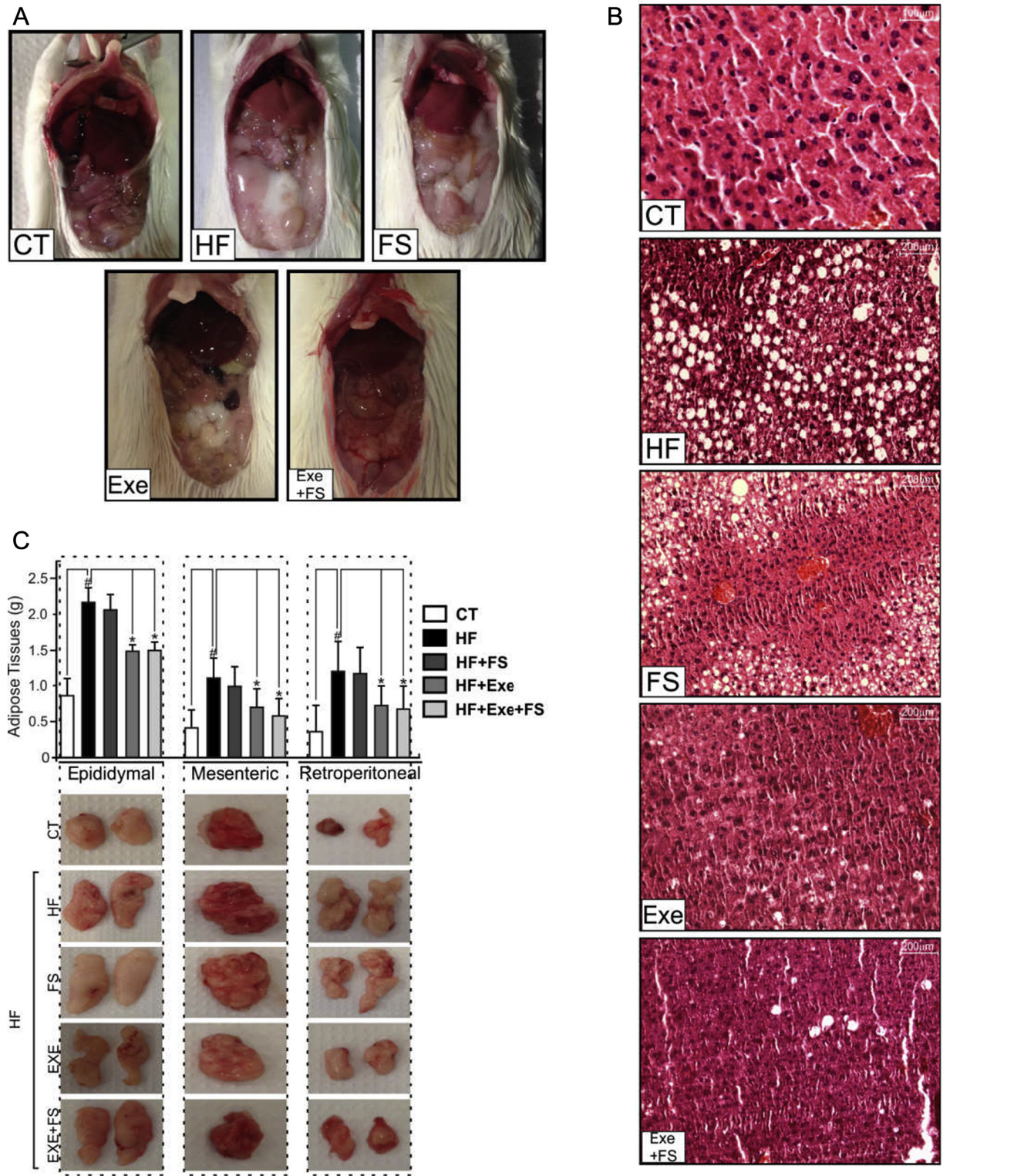


Fig. 2. The macroscopic appearance of liver and adipose tissue. (A) Liver and (C) adipose tissues (epididymal, mesenteric and retroperitoneal). (B) Histological evaluation (hematoxylin–eosin staining of 4-mm sections) of liver from CT, HF, HF + Exe, HF + FS or HF + FS + Exe groups. $^{\#}P<.05$ *vs.* CT (Student's test) and $^{*}P<.05$ *vs.* HF, HF + FS, HF + Exe and HF + Exe + FS (Tukey's test). CT, regular chow; HF, high-fat diet; FS, flaxseed oil; Exe, exercise training.

Fragments (~3 mm^3) of hepatic tissue were removed and immediately homogenized in extraction buffers with a Polytron homogenizer (PT 10-35 GT-Kinemática) kept at 4°C during this period. Total protein was quantified using the Bradford method [20]. The samples were then applied to a polyacrylamide gel for separation by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. At this moment, the membrane was stained with Ponceau to assure the quality and originality of the results. The resulting blots were blocked with 5% dry milk at room temperature for 1 h and then incubated with specific antibodies (see section 2.4, method). Specific bands were labeled by chemiluminescence, and visualization was performed on a fluorescence imaging system (G:BOX Chemi XRQ, Syngene, USA). The bands presented in the blots were quantified using the software UN-SCAN-IT.

2.8. Immunoprecipitation analysis

For immunoprecipitation analysis, 1.0 mg of total protein for liver homogenates was immunoprecipitated with 10 μl of anti-Barrestin2 using Protein A sepharose beads (GE Healthcare Life Sciences). Precipitates were then analyzed by a Western blot with anti-GPR120 and reprobed with mouse anti-βarrestin2.

2.9. RNA extraction and real-time PCR

A separate liver fragment (~3 mm^3) was removed and immediately homogenized with a Polytron homogenizer (PT 10-35 GT-Kinemática) using Trizol buffer (Life Technologies). The reverse transcription was performed as previously described [21]. The primers for *Gpr120* and *Gapdh* were obtained from ThermoFisher Scientific. Real-time PCR analysis of gene expression was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems).

2.10. Liver histology

A separate liver fragment (~3 mm^3) was removed and immediately maintained at formaldehyde 4% solution for 2 days. Afterward, the fragment was dehydrated with ethanol, cleared with xylene, embedded in paraffin wax (Histosec, Merck, Germany) and cut into 4-μm sections (Olympus microtome) [21]. Sections were mounted and stained with hematoxylin and eosin. The analysis and documentation of the sections were performed using a Leica FW 4500 B microscope.

2.11. Lipidomics

To test the ω3 incorporation by liver, mass spectrometry was used. Liver samples were submitted to sorptive tape-like extraction laser desorption ionization coupled with mass spectrometry imaging with direct stamping onto a silica gel (60 Å) plate for thin layer chromatography (Merck, Germany), as described previously [22]. The metabolic fingerprint of free fatty acids was performed using a MALDI-LTQ-XL instrument with a tissue-imaging feature (Thermo Fisher, San José, CA, USA). Data acquisition for the survey scan was performed at the *m*/*z* range of 150–600 in the negative ion mode. No matrix was applied.

2.12. Gas chromatography for the evaluation of FS oil composition

FS was chosen as the source of ω3. To test the quality of the FS oil and to certify the presence of the ω3 α-linolenic fatty acid, the methyl esters were separated by a DB-23 capillary column in a gas chromatograph (GC-6850 Series Gas Chromatography System, Agilent Technologies, Santa Clara, CA, USA) [23].

2.13. Statistical analysis

All results were first submitted to the Kolmogorov–Smirnov test to check for normality. A Student's *t* test was applied for the comparison of CT and HF groups. When appropriated, analysis of variance was used to compare three or more groups. Mean values ± S.D. were compared using Tukey's test. $P<.05$ was accepted as statistically significant in all cases.

3. Results

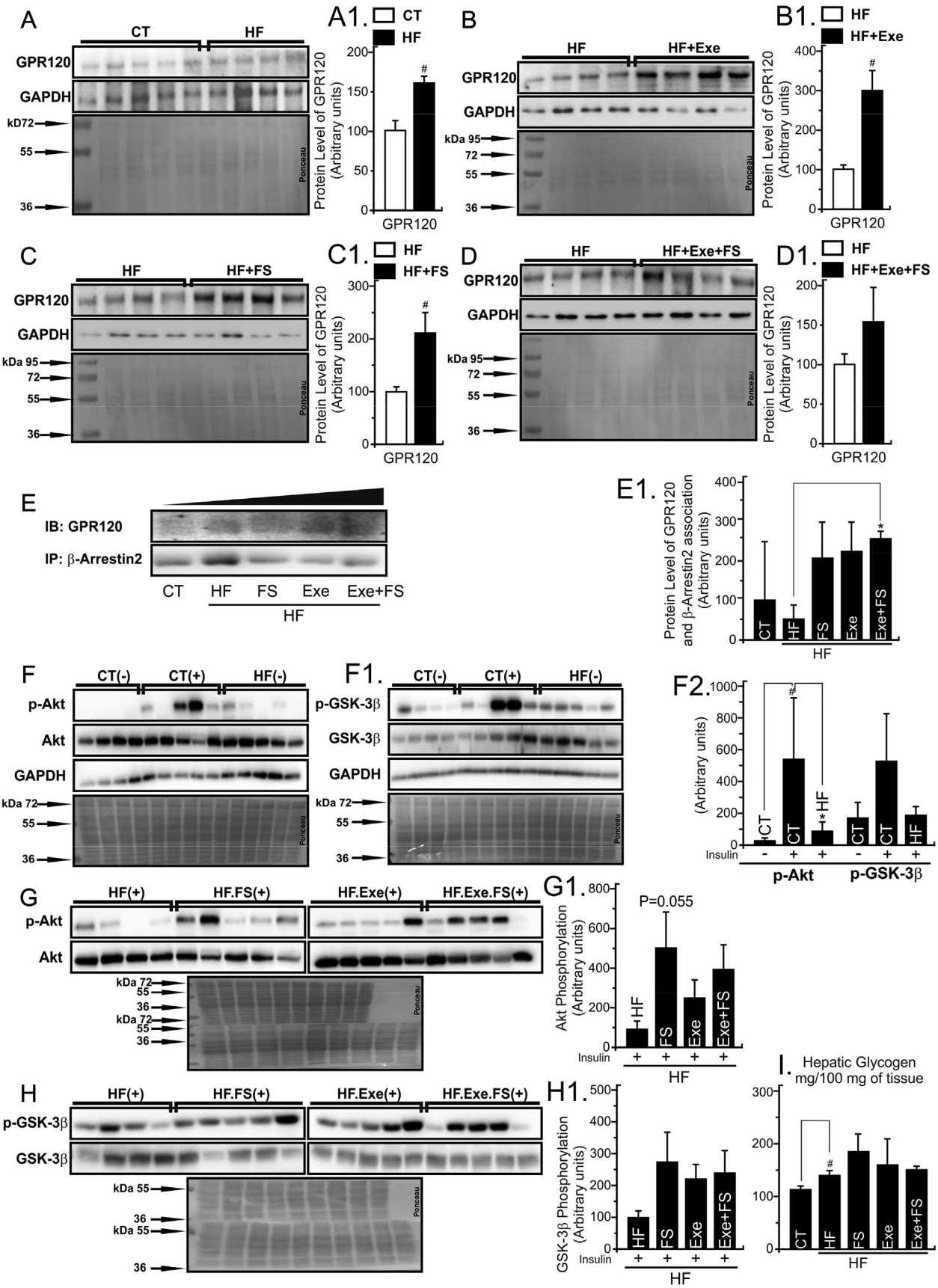
3.1. GPR120 expression after acute physical exercise in the liver of lean mice

Acute physical exercise significantly increased *Gpr120* gene expression in the liver at both 24 and 48 h post exercise compared to sedentary controls (CT) (Fig. S1B and C). The 24-h postexercise time point was therefore selected for liver extraction in subsequent experiments.

3.2. Acute exercise, but not FS oil, improves insulin signaling

We carried out a time-course test to understand how long it takes for ω3 to reach the blood stream (Fig. S2C) and activate the GPR120 receptor in the liver of mice (Fig. S2A). Based on these results, 500 μl of FS oil was administered by gavage 21 h after an acute exercise session. Two and a half hours after FS oil administration, glucose was injected i.p. (glucose 25%, 2 g/kg), and 30 min later, the liver fragment was removed (totaling 24 h postexercise time point) (see experimental design, Fig. S2B). Exercise alone (Exe) did not acutely change GPR120 protein levels but did increase Akt (3.45-fold) and GSK3 (4.9-fold) protein levels compared to controls (CT) (Fig. S2D). An injection of glucose following exercise (Exe + Gluc) further increased Akt phosphorylation (2.09-fold) compared to exercise alone (Exe) (Fig. S2E), but the administration of FS oil with or without glucose (Exe + FS or Exe + FS + Gluc) did not change GPR120 protein levels or Akt and GSK3 phosphorylation (Fig. S2F and G). Liver glycogen content was unaffected by any treatment (Fig. S2H).

Fig. 3. GPR120 expression in liver of mice after treatments. Twenty-four hours following the last exercise session and 12 h following the last FS oil treatment, extracts obtained from liver of Swiss mice fed on regular chow (CT), high-fat (HF), HF plus exercise training (HF + Exe), HF plus FS oil (100 μL) (HF + FS) or HF plus exercise training plus FS oil (100 μL) (HF + Exe + FS) were used in immunoblotting and immunoprecipitation experiments to evaluate GPR120 protein content or activity. A specific antibody against GPR120 was used to identify the protein target (A, B, C, D and E), with A1, B1, C1, D1 and E1 representing a quantitative representation of these blots. To test GPR120 intracellular signaling, β-arrestin 2 protein was immunoprecipitated with GPR120. The nitrocellulose membrane was immunoblotted against GPR120 antibody (E and E1). To test insulin signal transduction, mice were anesthetized 24 h after their last exercise session and acutely treated through portal vein with saline (100 μl) (−) or insulin (100 μl 10^{-6} mol/L) (+). After 30 min, fragments were obtained and used in immunoblotting experiments, blotted with phospho-Akt (F and G) or phospho GSK-3β (F and F1). Loading was evaluated by reprobing the membranes with GAPDH (A, B, C, D, F and F1), Akt total (F and G) and GSK-3β total (F1 and H). Ponceau staining was adopted to improve and guarantee the quality of Western blot membrane running. (I) The glycogen content in liver of animals in all groups was analyzed at the end of experimental period ($n=7$ for each group). The Western blot image bands displayed are original, and the gel was not cut. Values are expressed as mean ± S.D. CT ($n=6$), HF ($n=7$), HF + FS ($n=8$), HF + Exe ($n=7$) and HF + Exe + FS ($n=8$). Mean significant difference between $^{\#}$CT (−) *vs.* CT (+); *CT (+) *vs.* HF (+); $^{\#}$CT *vs.* HF; $^{\#}$HF *vs.* HF + Exe, HF + FS and HF + Exe + FS groups by Student's *t* test ($P<.05$). GPR120, G protein-coupled receptor 120; CT, regular chow; HF, high-fat diet; FS, flaxseed oil; Exe, exercise training; kDa, kilodalton.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FS
Exe
Exe+FS
HF
F
CT(-)
CT(+)
HF(-)
p-Akt
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GAPDH
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36
Ponceau
F1.
CT(-)
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Exe
Exe+FS
Insulin
HF
I.
Hepatic Glycogen mg/100 mg of tissue
CT
HF
FS
Exe
Exe+FS
HF

3.3. Exercise training improves insulin sensitivity and reduces body fat in obese and insulin resistant mice

Prior to initiating the chronic exercise, obesity, insulin resistance and glucose intolerance were induced by HF diet (Fig. S3A–F). Neither HF + Exe nor HF + FS oil treatment altered food intake compared to those on the HF diet alone (Fig.1B). The HF group maintained a higher weight than CT throughout the experimental period (Fig. 1C and D), and although there were no significant differences in body weight among the treated groups, at the end of experimental period, mice subjected to HF + Exe + FS treatment did have a lower body weight than HF alone (Fig. 1E). Fasting blood glucose levels were reduced in both HF + Exe and HF + Exe + FS groups, 18.61% and 21.2%, respectively (Fig. 1F), and the constant of glucose decay (K_{ITT}) improved 211.4% and 146%, respectively, compared to HF alone (Fig. 1G and H). K_{ITT} sensitivity was also around 110% higher in the HF + FS group compared to the HF group, but this did not reach significance (Fig. 1G and H). An HF diet led to a yellowish, hypertrophic liver with alterations in the macroscopic appearance. This was reverted to a normal condition on both the HF + Exe and HF + Exe + FS groups (Fig. 2A and B), with exercise, but not HF + FS, responsible for these changes. Both HF + Exe and HF + Exe + FS groups avoided fat accumulation in adipose tissue depots (epididymal, mesenteric and retroperitoneal) compared to HF alone (Fig. 2C).

3.4. HF diet, exercise training and treatment with ω3 from FS oil increase GPR120 levels in obese mice

Mice in the HF group showed a significant increase (59%) in hepatic GPR120 protein content compared to CT (Fig. 3A and A1), levels which were further increased by both exercise (HF + Exe) and FS oil (HF + FS) (196.73% and 112.9%, respectively) (Fig. 3B, B1, C and C1). However, the combination of both exercise and FS oil (HF + Exe + FS) failed to significantly increase GPR120 levels from the HF group (Fig. 3D and D1). Exposure to an HF diet, FS oil supplementation, or exercise training did not alter hepatic GPR40 levels (Fig. S4A–D).

3.5. Synergy between exercise training and ω3 from FS oil activates the GPR120 receptor and its intracellular cascade in obese mice

As presented above (3.4), both HF + Exe and HF + FS were capable of increasing GPR120 gene expression and protein levels, but we also wanted to assess GPR120 intracellular signaling to see whether the maximal benefits of ω3 were being obtained by this pathway by immunoprecipitating GPR120 with its first downstream protein βarrestin2. Both HF + Exe and HF + FS were capable of increasing the immunoprecipitation of GPR120 and βarrestin2; however, only HF + Exe + FS synergy increased with significance ($P<.05$) compared to HF alone (Fig. 3E, E1).

3.6. The effects of exercise training and ω3 from FS oil on insulin signaling

An HF diet alone was able to induce insulin resistance in the liver, with levels of Akt and GSK3 phosphorylation 528% and 182.6% lower than CT, respectively (Fig. 3F, F1 and F2). The HF + FS group showed an increased level of Akt phosphorylation (456.7%) from the HF group, but this did not reach significance ($P=.055$) (Fig. 3G and G1x). Likewise, there were no significant changes in Akt or GSK3 phosphorylation (Fig. 3G–H1) or glycogen hepatic content (Fig. 3I) among treatments compared to HF group alone.

3.7. Exercise training, FS oil or their combination reduces diet-induced inflammation

Mice on an HF diet showed an increase in the number of hepatic inflammatory markers compared to those on a chow diet (CT group) (Fig. 4A–F). The HF+ FS reduced TNFα protein levels and JNK phosphorylation (Fig. 4A and B), and the HF + Exe led to a decrease in TNFα and IL1β protein levels, and JNK and IκBα phosphorylation (Fig. 4A–E) ($P<.05$). The combination of HF + Exe + FS led to a decrease in TNFα and IL1β levels and JNK phosphorylation compared to HF alone (Fig. 4A, B and E) ($P<.05$).

3.8. FS oil improves the physical performance of mice

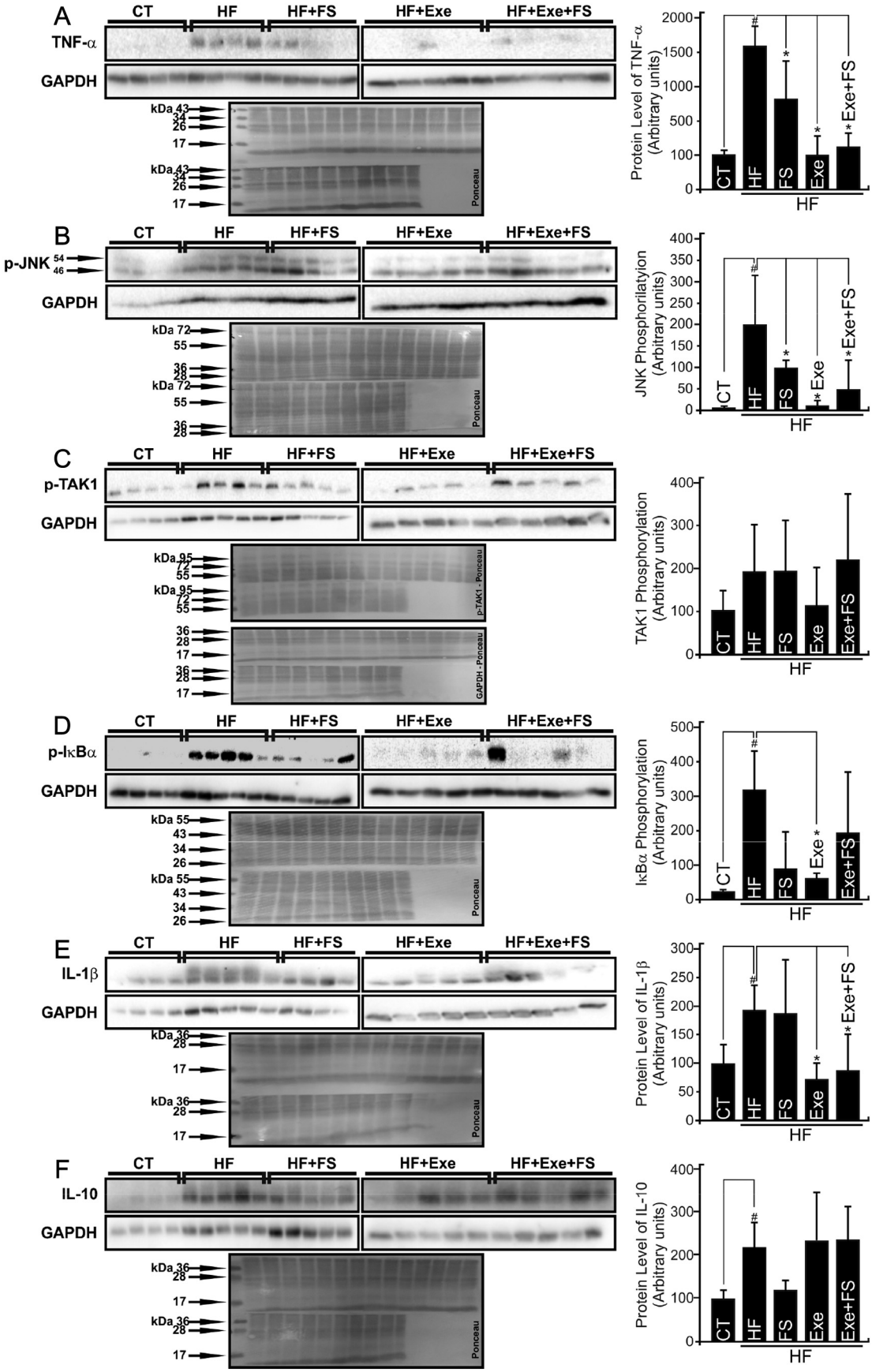
Before the beginning of the exercise training, in order to individualize the training workload for each mouse in their groups, the maximum potency (Pmax), distance ran and time spent on treadmill were assessed (Fig. 5). At the end of training, 4 weeks later, a 13.5% increase in running distance and an 11.5% increase in running speed were observed compared to before training, indicating an overall improvement in their performance (maximum potency), although their time to exhaustion did not increase (Fig. 5B and C). The HF + Exe + FS group significantly improved in all parameters across the training period, with increases in distance ran (26.9%), potency (12.5%) and, contrary to the HF + Exe group, running time (12.1%) (Fig. 5A–C). A direct comparison in the running parameters after training between the HF + Exe and HF + Exe + FS groups suggested that animals treated with HF + FS had a greater overall performance, with an increased time to fatigue and increased distance ran, and markedly, they ran at a higher intensity (Fig. 5D).

4. Discussion

Low-grade inflammation is considered one of the most relevant mechanisms of obesity and related disturbances. It is well documented that both ω3 supplementation and physical exercise have anti-inflammatory properties in obesity as well as in improving the action of insulin [24,25]. In this context, we focused on the association between dietary ω3 and exercise in obesity and show for the first time that chronic exercise and ω3 have a synergistic effect on the hepatic levels and anti-inflammatory signaling of the recently deorphanized GPR120, and improve metabolic and molecular parameters in obese mice.

In the present study, we initially assessed whether acute physical exercise in lean mice could modulate the expression of hepatic GPR120 and GPR40 but showed no changes in either receptor at the gene or protein level (data not shown). This is in agreement with a recent study where GPR120 was shown not to be involved in the regulation of energy metabolism in lean mice during an acute physical exercise session on a treadmill [26]. Additionally, Nishinaka et al. [27] were also unable to alter GPR40 expression with acute exercise in the hippocampus of depressed mice.

Fig. 4. The effect of chronic Exercise and FS oil on inflammation. Extracts obtained from liver of Swiss mice fed on regular chow (CT), high-fat (HF), HF plus FS oil (100 μl) (HF + FS), HF plus exercise training (HF + Exe) or HF plus exercise training plus FS oil (100 μl) (HF + Exe + FS) were used in immunoblotting experiments to evaluate protein expression or activity. Specific antibodies against tumor necrosis factor alpha (TNF-α) (A), phospho c-Jun N-terminal kinase (JNK) (B), phosphorylated transforming growth factor activated kinase 1 (TAK1) (C), phosphorylated NF-κB inhibitor alpha (IκBα) (D), interleukin 1 beta (IL1β) (E) and interleukin 10 (IL10) were used to assess respective protein levels. Loading was evaluated by reprobing membranes with GAPDH (A, B, C, D, E and F). Ponceau staining was adopted to improve and guarantee the quality of Western blot membrane running. The Western blot image bands used are original, and the gel was not cut. Values are mean ± S.D. CT ($n=6$), HF ($n=7$), HF + FS ($n=8$), HF + Exe ($n=7$) and HF + Exe + FS ($n=8$). $^{\#}P<.05$ *vs.* CT (Student's test) and $^{*}P<.05$ *vs.* HF, HF + FS, HF + Exe and HF + Exe + FS (Tukey's test). CT, regular chow; HF, high-fat diet; FS, flaxseed oil; Exe, exercise training; kDa, kilodalton.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IL-10
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Protein Level of IL-10 (Arbitrary units)

Next, we assessed the hepatic GPR120 and GPR40 levels in the liver of obese mice after 4 weeks of exercise training and a FS oil intervention. Of note was that the HF diet *per se* significantly increased the level of GPR120 compared to lean control (CT), which has previously been observed [28–31], and levels were also increased both by chronic exercise and FS oil treatment. The mechanisms by which physical exercise modulates GPR120 expression have not been investigated. However, the GPR120 modulation induced by HF was recently indicated by Chen et al. [32], in which they observed that Gpr120 gene expression is under the control of the transcription factor Cebpβ. They verified an increase in Cebpβ gene expression with an HF diet, which could therefore serve as a potential candidate behind GPR120 up-regulation in our study. The observed increase in GPR120 with FS oil is also in agreement with previous studies, with ω3-supplemented diets shown to elevate GPR120 expression in animals [33] and in children diagnosed with NAFLD [34]. Here, mice subjected to the exercise training plus the FS oil treatment did not present any further increases in GPR120 compared to HF + Exe, HF + FS or HF alone.

GPR40 levels were not changed by any treatment. GPR40 has the same agonists as GPR120 but only a 10% homology [35] despite using the same intracellular signaling cascades [7]. Previous data on the ability of exercise to modulate G protein coupled receptors in general are scarce. The long-chain fatty acids such as ω3 and ω9 are well-recognized GPR40 agonists [5]. Once activated, GPR40 could contribute against proinflammatory signaling in several tissues, increasing the insulin sensitivity and hence glucose uptake [4].

We also evaluated the effect of exercise training and FS oil on insulin action and glucose homeostasis, with both exercise alone and exercise with FS oil improving levels similar to those observed in lean controls. This is in line with previous studies that have shown that both acute physical exercise and chronic physical exercise are able to improve insulin action in obese mice [3,24,36]. We did not observe any effect of FS oil alone on insulin action. This is in line with a study where FS oil supplementation did not affect glucose control in individuals with well-controlled type 2 diabetes [37]. Surprisingly, physical training, FS oil or their combination did not have a significant effect on Akt or GSK3 activity, as some studies have shown [38,39]. We attributed our negative findings here to high variance in the group given the observed improvements in insulin sensitivity and glucose levels.

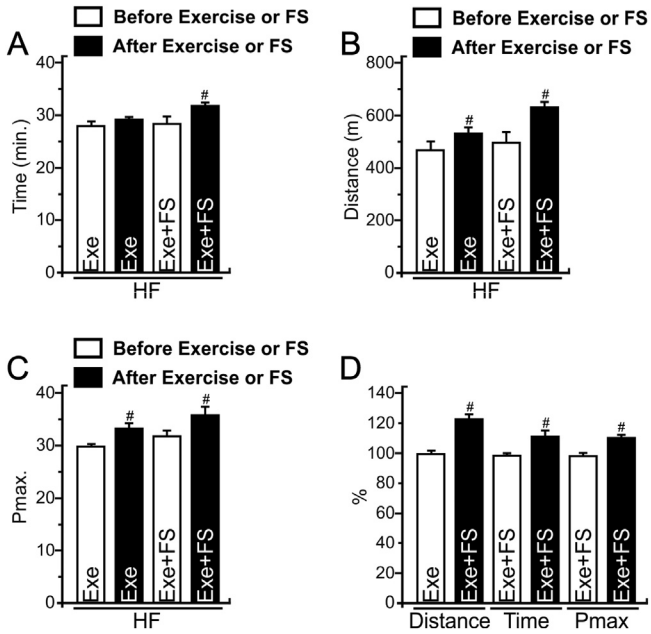

Fig. 5. Incremental exercise testing after chronic exercise and FS oil treatment. Mice were submitted to a previously described protocol of incremental exercise testing with and without FS oil, and their final performances were compared both within groups (compared to their initial performance) and between groups for (A) running time, (B) distance and (C) maximum potency. $^{\#}P<.05$ *vs.* Exe or FS (Student's test).

Overall, the role of FS oil in insulin signaling needs to be further investigated, with, notably, attention paid to the standardization of dose. For example, in the abovementioned where diabetic patients received FS oil, 13 g of the oil, totaling 7.4 g of alpha-linolenic fatty acid, was administered per day [37]. This is considered a very high dose, and this excess could be harmful, with our group previously showing that high levels FS oil in rodent diets (achievable only through supplementation) worsened several metabolic and molecular parameters, triggering a proinflammatory signaling [7]. Animal studies have also been inconsistent in FS oil dosage, with, for example, Bashir et al. [40] treating obese and diabetic mice with FS oil at 4 mg/kg, while Zhao et al. [41] used a diet containing 10% of FS oil to treat mice. This latter value corresponds to approximately 500 mg/day [41], and in our dosage studies, we determined the maximal safe dose to be at around 290 mg/day, which was easily achievable by diet alone. In our current study, we used 50 mg/day, which might not have been high enough to change the main pathophysiological parameters of obese mice despite the observed reduction of some proinflammatory proteins and the increase in GPR120 receptor levels in the liver of treated animals. Beyond dosing concerns, oil quality and the percentage of alpha-linolenic fatty acid in the oil can also cause difficulties to be reached. Generally, the percentage of ω3 in FS oil is around 58% [42], with a value of 52% obtained in this study, although levels as low as 33% have been used in other studies [43]. We therefore recommend that dose/response experiments to establish a minimal acceptable percentage of alpha-linolenic fatty acid in FS oil are necessary.

In our current study, we also investigated a number of inflammatory markers after the exercise training and FS oil interventions. As expected from the literature, both treatments presented a consistent reduction in inflammatory markers. Exercise is well described as one of the most important nonpharmacological anti-inflammatory strategies, shown to reduce TNFα, IL1β, IL6, IκK and IκBα, among others [3,44–46]. ω3 fatty acids induce the same pattern but through different mechanisms. As mentioned, an interesting research showed a coupling between GPR120 receptor and βarrestin2, an intracellular protein that disrupts the inflammatory signal transduced from TLR2/4 and TNF-α receptors [5]. Docosahexaenoic (DHA) and eicosapetaenoic (EPA) acid, and with a lower affinity alpha-linolenic (ALA) acid, activate this receptor and mediate the anti-inflammatory signaling [5], with the same molecular cascade observed across multiple body tissues [4,7]. The association between exercise and FS oil reduced the proinflammatory markers, however without further improvement to the anti-inflammatory response, probably through the same pathways above described.

We also investigated fat depots in mesenteric and retroperitoneal adipose tissues as well as in the liver and verified a reduction in lipid droplets following exercise or exercise and FS oil treatments in the liver from obese mice. Potential molecular candidates in the modulation of adipose tissue by exercise include irisin, which is secreted upon muscle contraction and can change the profile of adipose tissues among other functions [47]. However, a reduced adipose tissue mass in either humans or animals after chronic exercise exposure is mainly attributed to the increase energy expenditure [48]. FS oil treatment did not lead to a reduced fat mass profile in our study, although this has previously been observed elsewhere with other studies demonstrating a reduction in fat storage and the number and size of adipocytes [4,7,43]. Here, we believe that the period of treatment (4 weeks) and our mild dose of FS oil were perhaps insufficient to change the fat depots in either liver or adipose tissue, and an extended treatment period as suggested by Baranowski et al. [43] might be required.

In our final experiment, we unexpectedly demonstrated that animals treated with FS oil had an increased performance in the incremental load test. Previous studies have been somewhat inconsistent in showing a beneficial effect of ω3 in this area, with no improvement observed in maximal aerobic power, anaerobic threshold or running performance in well-trained soccer players supplemented for 10 weeks with 2.64 g of ω3 (1.6 g of EPA plus 1.04 g DHA) [49]. However, improvements in neuromuscular function, maximal voluntary isometric contractions, performance and fatigue levels were observed elsewhere in athletes after supplementation with 1.1 g of ω3 (375 mg EPA, 230 mg docosapentaenoic acid, 510 mg DHA) [50]. The translation of the current supplementation model to the human application is reasonable, once the ω3 (ALA) adequate intake is 1.6 g/day [51], which could be achievable with 3 mL of FS oil or 7 g of flaxseed.

Overall, although we do not demonstrate the ability of a FS oil supplement to revert obesity or insulin sensitivity beyond that of chronic exercise, we hypothesize that a longer treatment time at our low dose might allow these affects to come about, and future research directions could lead towards these modifications. Next explorations could determine how chronic exercise increases the GPR120 gene expression and protein content in the liver or different tissues.

In summary, our results show that acute physical exercise is not involved in the modulation of GPR120 or GRP40 expression in the liver of lean mice. On the contrary, we show for the first time that chronic exercise increased levels of GPR120, although not GPR40, in the liver of obese mice, as did an FS oil supplement. The insulin signaling was not ameliorated by interventions; however, the inflammatory tonus in the liver was improved. FS oil contributed to increase the performance of running mice, improving the aerobic power. These associated factors, for a longer time, could contribute as a new strategy against inflammation disorders associated to obesity, providing new insights in the study of GPR120.

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Disclosure statement

The authors declare that they have nothing to disclose.

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