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Physical exercise: preventive and therapeutic non-pharmacological intervention against non-alcoholic steatohepatitis?

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CONTENTS

LIST OF ABBREVIATIONS

11β-HSD1 - 11β-hydroxysteroid dehydrogenase type 1

AMLN - Amylin diet

ALT - Alanine aminotransferase

AP1 - activator protein 1

ASK1 - Apoptosis signaling-regulating kinase 1

AST - Aspartate aminotransferase

ATF - Activation transcription factor

ATF-4 - Activating transcription factor 4

ATF-6 - Activating transcription factor 6

BiP - Binding immunoglobulin protein

Caspase - Cysteine-dependent aspartate specific protease

CCl4 - Carbon tetrachloride

CCR - C-C Motif chemokine receptor

CDAA - Choline-deficient, L-amino acid-defined

CHOP - C/EPB homologous protein

CIAFEL - Research Centre in Physical Activity, Health and Leisure

eIF2α - Eukaryotic initiation factor 2 **α**

ER - Endoplasmic reticulum

ET - Endurance training

FADEUP - Faculty of Sport, University of Porto

FCT - Fundação para a Ciência e Tecnologia

FFAs - Free fatty acids

FMUP - Faculty of Medicine, University of Porto

FXR - Farnesoid X receptor

GSH - Reduced glutathione

GSx – Total (oxidized plus reduced) glutathione

HFD - High-fat diet

IκB - NF-κB inhibitor

IKK - IκB kinase

IL-1β - Interleukin-1 beta

IL-6 - Interleukin-6

List of abbreviations

IRE - Inositol requiring enzyme

IRE1α - Inositol requiring enzyme 1 α

IRS-1 - Insulin receptor substrate 1

IRS-2 - Insulin receptor substrate 2

JNK - C-Jun N-terminal kinase

LLPA - Life-long physical activity

MCD - Methionine and choline-deficient diets

Mc4r - Melanocortin 4 receptor knock-out

NAFLD - Non-alcoholic fatty liver disease

NASH - Non-alcoholic steatohepatitis

NF-κB - Nuclear factor κ-light-chain enhancer of activated B cells

Nrf2 - Nuclear erythroid 2 p45-related factor

ORA - Original research article

PBA - 4-Phenyl butyric acid

PE - Physical exercise

p-eIF2α - Phosphorylated eukaryotic initiation factor 2 α

p-JNK - Phosphorylated c-Jun N-terminal kinase

PERK - Protein kinase RNA-activated (PKR)-like ER kinase

PPAR - Peroxisome proliferator-activated receptors

PTP1B - Protein tyrosine phosphatase 1B

ROS - Reactive oxygen species

SD - Standard diet, standard control liquid diet

sXBP1 - spliced XBP1 X-box binding protein 1

T2DM - Type 2 diabetes mellitus

TAA - Thioacetamide

TGF-β - Transforming growth factor β

TGs - Triglycerides

TNF-α - Tumor-necrosis factor α

TUDCA - Taurine-conjugated ursodeoxycholic acid

UPR - Unfolded protein response

uXBP1 - unspliced X-box binding protein 1

List of abbreviations

VPA - Voluntary physical activity

XBP1 - X-box binding protein

RESUMO

O estilo de vida sedentário associado ao consumo excessivo de alimentos hipercalóricos está relacionado com o aumento epidémico da esteatose hepática não alcoólica (*non-alcoholic fatty liver disease, NAFLD*). Esta pode evoluir de simples esteatose para esteatohepatite não alcoólica (*non-alcoholic steatohepatitis, NASH*), fibrose e cirrose, culminando, eventualmente, em carcinoma hepatocelular. Embora os mecanismos subjacentes não estejam ainda completamente compreendidos, a lipotoxicidade associada à deposição de gordura, o stresse oxidativo, o stresse do retículo endoplasmático, a inflamação, a disfunção mitocondrial, a resistência à insulina e a apoptose estão implicados no seu surgimento e na sua subsequente progressão. Estes mecanismos hepáticos interligam-se num ciclo vicioso.

Não existe ainda terapia farmacológica aceite para o tratamento da NASH. Atualmente, o exercício físico apresenta-se como uma intervenção não-farmacológica efetiva, não só preventiva como também terapêutica, que mitiga diversas alterações idênticas às que se observam na NASH. No entanto, os efeitos benéficos do exercício físico nos mecanismos acima mencionados num contexto de NASH são ainda pouco compreendidos.

Com esta dissertação, que integra um artigo de revisão e três trabalhos experimentais originais, pretendeu-se avaliar alguns mecanismos moleculares potencialmente subjacentes aos efeitos benéficos do exercício físico num modelo animal de NASH: ratos machos adultos Sprague-Dawley alimentados com uma dieta líquida rica em gordura (Lieber-DeCarli), durante 17 semanas. Assim, foram analisados os efeitos da atividade física voluntária (*voluntary physical activity*, VPA, durante 17 semanas) e do treino de resistência (*endurance training, ET*, durante as últimas 8 semanas da intervenção alimentar), estratégias preventivas e terapêuticas, respetivamente, em a) marcadores antropométricos, assim como histológicos (no fígado) e metabólicos (no plasma) **(trabalho experimental original 1)**, b) marcadores pró-inflamatórios e de sinalização pró-inflamatória assim como da sinalização pela insulina e por glicocorticóides **(**no fígado, **trabalho experimental original 2)**, e c) marcadores do stresse do retículo endoplasmático, do stresse oxidativo e da apoptose **(**no fígado, **trabalho experimental original 3)**.

Os resultados suportam a sugestão de que o treino de resistência possa ser considerado uma estratégia terapêutica não farmacológica para contrariar não só a deposição de gordura, mas também a inflamação, induzidas pela obesidade, uma vez que reverteu todas as alterações anatómicas e clínicas/histológicas observadas na NASH. Além disso, o exercício físico modulou positivamente a) a sinalização pelo JNK, bem como os níveis de IL-1β, IL-6, LIF e TGF-β e b) a sinalização pela insulina, provavelmente através da sinalização pelo JNK, PTP1B e 11β-HSD1. Além disso, o exercício físico modulou positiva e dinamicamente a UPR (aumentando a resposta pró-vida, enquanto regula negativamente a pró-morte) pela via de sinalização IREα/XBP1.

Em conclusão, o exercício físico deve ser considerado uma estratégia não farmacológica na prevenção e/ou tratamento da NASH.

ABSTRACT

An inactive lifestyle in simultaneity with excessive consumption of high-calorie food associates with the global increase of non-alcoholic fatty liver disease (NAFLD). NAFLD evolves from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Hepatocellular carcinoma can be end-stage of NAFLD. Although the underlying mechanisms are not yet fully understood, lipotoxicity from fat deposition, inflammation, insulin resistance, oxidative and endoplasmic reticulum stresses, mitochondrial dysfunction, and apoptosis are implicated in its onset and subsequent progression. These hepatic mechanisms are linked through vicious cycles.

Currently, physical exercise is an effective non-pharmacological intervention, not only preventive but also therapeutic, which can mitigate several alterations identical to those observed in NASH. However, the beneficial impact of physical exercise on the aforementioned mechanisms in a context of NASH is still poorly understood.

With this dissertation, which integrates one review article and three original research articles, it was intended to evaluate some molecular mechanisms potentially underlying the beneficial impact of physical exercise in an animal model of NASH: adult male Sprague-Dawley rats fed a liquid high-fat diet (Lieber-DeCarli), for seventeen weeks. Thus, we analyzed the effects of voluntary physical activity (VPA, during seventeen weeks) and endurance training (ET, during the last eight weeks of the dietary intervention), preventive and therapeutic strategies, respectively, in a) anthropometric markers as well as histological (in the liver) and metabolic (in the plasma) markers (original research article 1), b) pro-inflammatory and pro-inflammatory signaling markers as well as insulin and glucocorticoid signaling (in the liver, original research article 2), and c) markers of endoplasmic reticulum stress, oxidative stress and apoptosis (in liver, original experimental work 3).

The results support the suggestion that endurance training could be considered a non-pharmacological therapeutic strategy to counteract both the development of hepatic lipid droplet accumulation and inflammation, since it reverted the anatomical and clinical/histological alterations observed in an animal model of HFD-induced NASH. Additionally, physical exercise positively modulated a) JNK signaling as well as IL-1β, IL-6, LIF and TGF-β and b) insulin signaling, most probably through JNK signaling, PTP1B and 11β-HSD1. Furthermore, physical exercise positively and dynamically

modulated the NASH-related UPR by enhancing pro-survival response, while down-regulating pro-death, through the IREα/XBP1 signaling pathway.
Altogether, physical exercise should be considered as a non-pharmacological strategy in the prevention and/or treatment of NASH.

CHAPTER 1: INTRODUCTION

INTRODUCTION

Nowadays, the world is facing a dramatic increase of obesity: an excessive consumption of high-calorie food accompanied by a sedentary lifestyle is responsible for this epidemic [1-5]. Obesity results from an excess of adiposity, which is widely accepted as the most significant risk factor for, among others, insulin resistance/type 2 diabetes mellitus (T2DM), cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD), along with the associated comorbidities [6-16].

NAFLD prevalence has drastically increased in the last decade, affecting up to 25% of the world's population [4, 17-21]. NAFLD denotes an umbrella term for a spectrum of hepatic histopathological changes (and their sequential progression) where benign simple steatosis (more than 5% of fat accumulation within hepatocytes), non-alcoholic steatohepatitis (NASH; steatosis plus hepatocellular inflammation and damage), fibrosis (can be present or absent in NASH), cirrhosis and hepatocellular carcinoma are included [17, 20-33]. As NASH correlates with features of the metabolic syndrome (i.e., obesity, insulin resistance, T2DM, dyslipidemia and hypertension) [16, 34-37], it has been considered a manifestation of the metabolic syndrome in the liver [38, 39]. NASH also associated with an increased risk for cardiovascular complications [38].

The "two hits" hypothesis (proposed in 1998) explained for the first time the development of NASH [40]. The "first hit" would be hepatocyte fat storage which would induce steatosis; the "second hit" would be increased hepatocyte oxidative stress which would stimulate hepatocyte lipid peroxidation [40]. Since NASH pathogenesis is complex and multifactorial, involving several parallel molecular and metabolic changes and converging signaling pathways [17, 33, 38], a more accurate "multiple hits" hypothesis has been proposed [17, 20, 33, 41-44] where several insults act synergistically, sometimes on a background of genetic predisposition, to promote NAFLD/NASH onset and its development/progression [27, 28, 30, 33, 38, 41, 45-51].

The "multiple hits" hypothesis includes, for example, lipotoxicity of the aberrant hepatic lipid accumulation, disturbed hepatic fatty acid and cholesterol metabolisms, altered hepatic tissue lipid composition and adipokine secretion, adipose and hepatic pro-inflammatory states, hepatic mitochondrial dysfunction, increased hepatic endoplasmic reticulum (ER) and oxidative stresses, impaired hepatic and adipose

glucocorticoid and insulin signaling pathways and increased hepatic cell death that converge towards the induction and/or increase of hepatic steatosis, inflammation and fibrosis and, consequently, in the onset of NAFLD and progression of NASH [4, 5, 9, 17, 19, 22, 31, 41, 42, 44, 51-71].

The "multiple hits" hypothesis for the onset of NAFLD and progression of NASH also considers liver interactions with other tissues, organs and systems, besides adipose tissue: intestine, gut microbiota and immune system, for example [17, 33, 42-44, 61-63, 72-82]

A tremendous effort is being done in the development of adequate/effective therapies able to target NASH features as a whole through the use of animal models. These models mimic the human disease aiming to a) completely characterize the full range of biological and environmental factors that drive the initiation of NAFLD and progression of NASH [17, 31, 53, 78], and b) serve as the preclinical platform to study therapeutic strategies for inhibiting NAFLD onset/NASH progression [4, 20, 21, 53, 83-87]**.**

Rodent models in NAFLD

Several animal species can be used to study NASH. Rodents are commonly chosen because they are adequate for drug testing, can be manipulated without difficulty and easily develop obesity, metabolic dysfunction, T2DM, and NAFLD [17, 88-90]. Nowadays, different animal models exist based on genetic alterations, chemical intoxication and/or dietary interventions [17, 53, 78]. These animal models are characterized by decreased hepatic lipid export and fatty acid oxidation, and/or increased hepatic lipogenesis and fat uptake [20, 91-93].

Genetic models

With genetic models is possible to identify the pathophysiological consequences of modifications in genes (and, consequently, specific mechanisms) most probably involved in the development of NAFLD [17, 88].

Single mutations of mice genes implicated in the regulation of appetite [especially in the models ob/ob (leptin-deficient), db/db (deficient leptin signaling), foz/foz (spontaneous mutations in the Alström syndrome 1 gene) and apolipoprotein E knock-out and knock-in] and knock-out mice for the melanocortin 4 receptor (Mc4r–/–) have been used to induce NASH [52, 78]. However, these mice disease models contrast with the human disease, where a background of obesity and metabolic dysfunction, adding to epigenetic modifications, various single-nucleotide polymorphisms, and/or environmental factors, contribute to the pathological condition [78]. It should be highlighted that genetic models present clear limitations in representing the human disease as they do not usually develop NASH, fibrosis or hepatocellular carcinoma spontaneously: special diets should be used to further progress from steatosis [17, 53, 78, 94-96].

Chemical models

A common procedure to induce the development of NASH is through the administration of toxic insults to the liver, such as thioacetamide (TAA), tetracycline, streptozotocin, and carbon tetrachloride (CCl4) [17, 88, 89]. Although the fastest and more powerful way to study liver disease is through the use of chemical models, its initiation and progression steps bear lesser resemblance to human NASH than with the dietary models, clearly differing in the etiopathogenesis process from human metabolic dysfunction obesity-associated NASH [53, 78]. The risk of studying NASH with chemical models is that we are more likely to evaluate the effects of the chemical itself than the impact of an obesity-induced impaired metabolism [78].

Dietary models

Since the genetic and chemical animal models possess limitations to mimic the human metabolic dysfunction obesity-associated NASH, it seems that animal NASH models should be ideally set up on a nutritional basis [53]. In fact, a high-fat and high-sugar diet is one of the major factors strongly associated with NASH development in humans [52]. Dietary models can include *deficient* or *high-amount* diets [17]. Feeding of methionine and choline-deficient diets (MCD) is one of the most commonly used

methods to induce the rapid onset of the histological phenotype of severe NASH, including fibrosis [17, 20, 39, 88, 89]. However, animals treated with MCD diet lose body weight and neither develop insulin resistance nor the associated comorbidities [20, 78, 88]. Additionally, the choline-deficient, L-amino acid-defined (CDAA) diet, which due to choline deficiency is similar to the MCD, not only induces a greater increase in the levels of alanine aminotransferase (ALT) but also promotes a slightly more severe degree of NASH than MCD [17, 78].

Diets most closely resembling the NASH-related human dietary habits are high-calorie diets, with an extremely high amount of certain nutrients [52]. High-fat diets (HFDs) are widely applied to induce NASH [4, 97-99]. HFDs may include a wide range of formulas with different types of fat (that comprise 30 - 71 kcal% fat) and occur in different forms (i.e., pellet or liquid) [20]. The Sprague-Dawley rat strain ingesting high-fat, liquid diet (71% of energy from fat, 11% from carbohydrates, 18% from protein) is the first animal NASH model showing panlobular steatosis and inflammatory infiltrate with predominantly mononuclear cells. After feeding with liquid HFD for 3 weeks, rats showed increased tumor-necrosis factor alpha (TNF-α), collagen type 1, α1(I) procollagen and 4-hydroxynonenal levels in the liver as well as plasma insulin levels [98].

Additionally, NASH can be induced with (i) high-fat and high-sugar diet, (ii) high-sugar diet, based on fructose or sucrose, (iii) high-cholesterol and cholate (atherogenic) diet, (iv) high-fat and high-cholesterol diet, as well as (v) *Western diet* or *fast food* diet and amylin diet (AMLN) [17, 20, 52, 78, 88, 89, 100-102].

Combined models

Since genetic models do not generally develop NASH and fibrosis, they must be given special diets to further progress from steatosis and to achieve the desired liver damage [17, 20, 52, 53, 78, 94-96, 103] and, consequently, to generate metabolic and histologic features of human NASH [88].

Lipotoxicity, inflammation and insulin resistance

Epidemiological correlations between obesity and NAFLD are well-established but the molecular mechanisms explaining them are not completely known and understood [21, 104]. In this regard, the *key switch* between obesity-induced hepatic steatosis and NASH remains to be discovered [27] and, even though hepatic fat accumulation is an essential event, currently, the concept of "multiple hits" is more appropriate [17, 20, 33, 41-44]. In fact, ectopic fat accumulation in the liver ensues when the fat storage capacity of adipose tissue is overwhelmed [21]. The generally accepted dogma is that hepatic triglycerides (TGs) accumulation occurs as a result of increased free fatty acids (FFA) flux from adipose tissue to the liver, increased intake of dietary fat, increased hepatic *de novo* lipogenesis, decreased hepatic β-oxidation, and/or decreased hepatic TGs secretion [21, 27, 28, 30].

Overall, adipose insulin resistance has been proposed as the prime contributor to excessive storage of hepatic TGs [29]. In this regard, an uninhibited lipolysis in adipose tissue, as a consequence of an impaired insulin metabolism, would lead to an increased flux of FFA to the liver, being visceral adipose tissue considered the major source of FFA to the liver due to the direct drain of its circulation to the portal vein [22, 45, 105]. However, lipid accumulation in hepatocytes, *per se*, is not necessarily dangerous because liver injury is determined by lipid quality rather than by accumulated lipid quantity [26], as the cytotoxicity of saturated fatty acids is higher than the cytotoxicity of unsaturated fatty acids [5, 106-109].

Lipotoxicity, a hallmark of NASH, includes chronic inflammation, abnormal activation of intracellular signaling pathways, organellar dysfunction, and higher/dysregulated cell demise induced by the toxicity of lipids [21, 110-117]. Lipotoxicity encompass several cellular processes, such as ER stress and mitochondrial dysfunction [113], leading to and/or increasing, among other features, inflammation [21], altered lipid composition [118, 119], and insulin resistance [21, 32, 113]. In fact, in a overnutrition setting, adipocytes become hypertrophic and initiate a state of cellular stress, altering the release of several hormones and adipokines which create a pro-inflammatory milieu [17, 120], first in the visceral adipose tissue and then in the liver [121]. In this regard, liver inflammation in NASH might originate in the visceral adipose tissue [5,

106, 122-124] and/or could be a response to visceral adipose tissue-induced hepatic lipotoxicity [125, 126].

Lipotoxicity activates the c-Jun N-terminal kinase (JNK) and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) signaling pathways in the liver [21]. Additionally, activation of hepatic JNK (as a consequence of exacerbated oxidative stress in the liver) increases the pro-inflammatory factors TNF-α and interleukin (IL)-6 [5, 67, 127, 128]. Hepatic inflammation mediated by NF-κB, NF-κB inhibitor (IκB) kinase (IKK) and/or JNK pathways, adding to hepatic ectopic lipid deposition and ceramide and diacylglycerol buildup, induces and/or exacerbates hepatic insulin resistance [55, 60, 110, 129-133]. Activation of IKK and JNK, particularly the JNK isoform1, disrupts the insulin signaling pathway through the inhibitory phosphorylation of insulin receptors substrate (IRS) 1 and IRS2 [5, 129, 131, 134, 135].

Additionally, published data suggest that increased expression of protein tyrosine phosphatase 1B (PTP1B) and 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays an important role in hepatic insulin resistance and inflammation [5, 50, 55, 105, 126, 136-138]. As both enzymes are abundantly located/tethered on the cytosolic surface of the ER [5, 55, 105], the ER functionality is implicated in the onset and/or progression of hepatic insulin resistance and inflammation. Likewise, increased signaling of hepatic transforming growth factor-β (TGF-β) strongly correlates with hepatic insulin resistance and contributes to hepatic inflammation, steatosis and fibrosis, what suggests TGF-β as a crucial player to the onset of NAFLD and the progression of NASH [55, 139-142].

Cross-talk between ER stress, inflammation, insulin resistance, altered redox state and mitochondrial dysfunction

Sustained ER dysfunction and consequent unmitigated activated unfolded protein response (UPR) seem to play an important role in the onset and progression of NASH [5, 12, 60, 143, 144], both in humans and animal models [145-147].

The mechanisms of the complex phenomenon of NASH-related ER stress are not completely understood and most of them are nonexclusive [67, 134, 143, 147-156],

with ER stress-associated hepatic inflammation, insulin resistance, oxidative stress, mitochondrial dysfunction and Ca^{2+} homeostasis disruption as well as apoptotic signaling involved in this process, in a vicious cycle manner [9, 67, 157-161]. Briefly, the dysfunction of the ER (the principal cellular location involved in secretory and transmembrane protein folding, lipid biogenesis and Ca^{2+} homeostasis) leads to the activation of an elaborate adaptive UPR [4, 5, 9, 60, 67, 128, 134, 152, 159] driven by three key ER transmembrane proteins [protein kinase RNA-activated (PKR)-like ER kinase (PERK), inositol requiring enzyme (IRE) 1 and activation transcription factor (ATF)], resulting in downstream responses [144, 153] that dynamically adjust the protein folding capacity to the cell needs [162] in order to restore homeostasis and to enhance cell survival. The first stage of UPR activation could be faced as a window of opportunity for the cell to restore homeostasis through expansion of the ER, decrease of protein synthesis, increase of the protein folding capacity and increase of the degradation of ER terminally misfolded proteins [4, 5, 114, 134, 153, 155]. However, prolonged ER stress might trigger and/or increase hepatic inflammation, insulin resistance, oxidative stress, mitochondrial dysfunction, Ca^{2+} homeostasis disruption and, ultimately, cell death [9, 67, 157-161], all of those accepted as NASH features.

The ER stress-induced unmitigated activation of UPR plays an important role in the inflammatory NASH-related response via phosphorylation of IκB by IKK as well as through the activation JNK/activator protein 1 (AP1) [5, 9, 67, 127, 134, 163, 164]. It is well-recognized that increased activation of the PERK and IRE1 pathways mediate increased expression of pro-inflammatory signaling, such as TNF-α, IL-1β and IL-6, through the activation of JNK, IKK and NF-κB [5, 110, 133]. Nevertheless, it should be noted that increased accumulation of inflammatory cytokines in the liver can also activate UPR [9, 127, 161], suggesting that the relationship between ER stress and inflammation is bidirectional.

Additionally, prolonged ER stress has been involved in the development of hepatic insulin resistance. Although the mechanisms by which it decreases insulin signaling are complex and take place at various and different levels of the insulin signaling pathway, it is widely accepted the involvement of PERK and/or IRE1 pathways activation in this process through the activation of JNK1, IKK and NF-κB [5, 67, 149, 150]. Another mechanism through which prolonged ER stress can induce insulin

resistance is through PTP1B and 11β-HSD1 [5, 105, 136, 165]. Both PTP1B and 11β-HSD1 have been suggested as one of the main negative regulators of insulin sensitivity, being PTP1B involved in dephosphorylation of tyrosine residues in IRS1 and IRS2, while increased glucocorticoid levels (which are regulated by 11β-HSD1) lead to increased phosphorylation of IRS1 at serine^{307} and, consequently, decreased affinity of IRS1 for the insulin receptor [5, 55, 105, 138, 166, 167]. In fact, the deletion and/or inhibition of 11β-HDS1 is linked with improved insulin sensitivity [55, 168]. Additionally, prolonged ER stress may also induce hepatic insulin resistance through hepatic ectopic lipid deposition [60].

Another major factor that has been implicated in the pathogenesis of NASH is increased oxidative stress [169], which is reinforced by the cross-talk between ER stress and redox state [128, 169]. Because ER and mitochondria are physically and functionally interconnected, and oxidative protein folding occurs in the ER, alterations in the redox state by increased generation of reactive oxygen species (ROS) could, directly and/or indirectly, interfere with ER homeostasis and protein folding capacity as well as influence mitochondrial function by increasing mitochondrial oxidative stress [128]. Moreover, increased mitochondrial ROS generation increases pro-inflammatory signaling [4, 55, 68, 170, 171]. In fact, increased ROS generation and increased pro-inflammatory signaling are closely tangled, with various vicious cycles occurring between them leading to insulin signaling dysfunction and fibrosis development through the production of TGF-β [4, 68, 169-171]. On the other hand, excessive lipid accumulation induces ROS production and lipid peroxidation, both of which are deeply related to hepatic inflammatory reactions [169, 172-174]. Additionally, since ROS generation also occurs during the protein folding process, an increased but untroublesome oxidative stress is a component of the UPR. ROS can be produced both downstream and upstream of UPR suggesting a strong cross-talk between increased ROS production and ER stress [169]. In fact, an increased ROS production as a consequence of mitochondrial dysfunction stimulates an ER stress response that, in turn, aggravates, through a vicious cycle mechanism, mitochondrial dysfunction and ROS production [5, 9, 161, 175]. Simultaneously, an enhanced ROS generation increases mitochondrial dysfunction. Furthermore, both increased oxidative damage and ER stress causes the leakage of Ca^{2+} from the ER triggering its

accumulation inside the mitochondria. This increases ROS production by the mitochondria what amplifies the ER stress [5]. It is noteworthy that the UPR can act as a *binary switch* depending on the severity and nature of the ER stress. So, UPR regulates adaptive and death effectors (controlling the fate of the cell: survive or die) [5, 176, 177]. Under conditions such as those observed in NASH, where ER stress in sustained and/or unmitigated, cell death occurs through several mechanisms, including: (I) PERK pathway; (II) IRE1-mediated activation of apoptotic signaling through both apoptosis signaling-regulating kinase 1 (ASK1)/JNK and caspase-12; (III) release of Ca^{2+} from the ER [5, 177-180].

Overall, in NASH, ER can play an important role in the amplification of the above harmful vicious cycles, as sustained or inadequate ER stress responses *per se* can lead to or exacerbate hepatic fat accumulation, pro-inflammatory processes, insulin resistance, oxidative stress, mitochondrial dysfunction and apoptosis.

Pharmacological and non-pharmacological therapeutic strategies: the role of physical exercise

Excessive hepatic fat accumulation *per se* does not lead to liver damage but lays the foundation for others factors, such as increased inflammation, altered redox state (by increased oxidative stress) or increased/prolonged ER stress, to trigger the progression towards NASH [181]. Efforts are being done in order to identify the mechanisms that mediate the development and progression of NASH, with several pharmacological target molecules proposed as therapeutic strategies to fight organelle-associated dysfunction [182-189]. In this regard, and since the ER stress is well-known to play a crucial role in “multiple hits” hypothesis of NASH progression, the ER stress chemical manipulation with exogenous chaperones [compounds with low-molecular-weight such as taurine-conjugated ursodeoxycholic acid (TUDCA), betaine and 4-phenyl butyric acid (PBA)] has been applied to improve the capability of the ER to adapt [151, 185, 186, 188] and, consequently, counteract NASH.

Although it is predicted that NASH may speedily become the most common cause of liver transplantation in many countries in the coming years, a well-established

pharmacological treatment capable to counteract the pathology of such disease is still lacking [20, 21, 29, 53, 83, 189]. Therefore, the core of innovative pharmacological research for the treatment of NASH relates to drugs that targets hepatic lipid metabolism, insulin signaling and inflammation as well as hepatocyte injury, liver fibrosis, bile acid metabolism, gut microbiota, gut permeability, hepatic oxidative stress and hepatic apoptosis, with some of these drugs already in phase II and III trials [4, 189, 190]. The inexistence of approved pharmacological therapies for NASH could be explained by the existence of gaps in the knowledge of the mechanisms underlying the disease as well as the absence of consistent methods for diagnostic and stadiation, except the liver biopsy [189].

For this reason, the pharmacological treatment for NASH consists in the use of different drugs for different therapeutic targets. Nowadays, insulin sensitizers and antioxidants are prescribed for NASH management. The most important classes of drugs currently under investigation are farnesoid X receptor (FXR) agonists, peroxisome proliferator-activated receptors (PPAR) agonists, ASK1 inhibitor, C-C motif chemokine receptor (CCR) 2/CCR5 dual inhibitor, pan-caspase inhibitor, *de novo* lipogenesis inhibitors, fibroblast growth factor 19 agonist, thyroid hormone receptor agonist, glucagon-like peptide 1 receptor agonists, antidiabetic drugs, anti-inflammatory drugs, anti-apoptotic drugs and anti-fibrotic drugs [36, 52, 189-196]. Unfortunately, only a few of them have had their efficacy confirmed in phase III trials, others haven't had their superiority confirmed *versus* placebo, and some others are still under investigation [189, 190].

There is a lack of national guidelines for the treatment of NASH patients and the existent reference guidelines are not widely used [197]. In line, a "Call to Action" has been created by numerous outstanding medical associations to issue guidance strategies for the management of NASH [198, 199].

Considering that the pharmacological agents tested thus far display limited efficacy and NASH is a disease with tremendous pathophysiological complexity, it seems reasonable to consider that, in a near future, drug approach could not be appropriate/enough and lifestyle interventions should be taken into account [4, 5, 189, 194, 200].

The complexity of NASH pathology results from problems caused by “multiple-hits”. Given that the simultaneity of over nutrition with an inactive lifestyle is the primary cause for the development of NAFLD/NASH [5, 17, 20, 33, 41, 49, 55, 201], in one hand, and the pleiotropic characteristics of the beneficial effects of PE, in another hand, it is conceivable, therefore, that exercise training could be considered as a non-pharmacological cornerstone strategy against NASH and NASH-associated metabolic disorders [5, 202-204]. Although the exact molecular mechanisms responsible for the beneficial effects of physical exercise (PE) against NASH and NASH-associated metabolic disorders are not completely elucidated, the suggested positive modulatory effects may occur at various and distinct levels [5]. In fact, even though there is strong evidence suggesting that a reduction of 5%–10% in baseline body weight, through lifestyle interventions, is needed to improve NASH histological findings [87, 205], PE has been repeatedly shown to improve hepatic steatosis, lipolysis in the adipose tissue and fatty acids flux to the liver, even in the absence of the above-mentioned body weight reduction [84, 85, 87, 205-207]. Likewise, PE also reduces liver fibrosis scores without body weight reduction, highlighting its importance in NASH [87, 207].

Additionally, regular exercise training improves hepatic insulin sensitivity [55, 208-211], probable as a result of the favorable modulation of JNK activation (through JNK phosphorylation levels), 11β-HDS1 and PTP1B expression and pro-inflammatory signaling (through NF-κB protein expression, for example) [5, 55, 168, 212-214]. Additionally, anti-inflammatory effects of PE have been described [4, 5, 55, 208, 214, 215].

Given that both increased pro-inflammatory signaling and oxidative stress can be induced by both prolonged ER stress and mitochondria dysfunction, and taking into account that the simultaneity of over nutrition with physical inactivity is the primary cause for the NAFLD/NASH development [5, 49, 55], it will not be a surprise if the protective phenotype induced by PE against the progression of NASH lays on the recovery of ER stress and mitochondrial functionality. Although several studies have analyzed the effects of PE on ER stress response, the response perpetrated by PE still remains obscure with apparent contradictory results (increasing UPR *versus* decreasing UPR), suggesting that the effects of PE in ER stress are tissue-specific, dynamic and work as a *binary switch*: suppressing death effectors and increasing the

protective and pro-survival response [4, 5, 99, 208, 216, 217]. The idea that the exercise training could be an efficient therapy against NASH-related ER stress prompt us to hypothesize that exercise training might be able to modulate the protective effects of the existent *windows of opportunity* in the first phase of UPR.

Regarding the effects of PE on redox state, a variety of studies described PE-induced beneficial redox changes, including improvement of antioxidant defenses, possibly, mediated by nuclear erythroid 2 p45-related factor (Nrf2) [5, 69, 166, 185, 218-220].

Although the preventive and therapeutic effects of exercise training against NASH features and NASH-related ER stress, including its regulatory mechanisms, are only scarcely understood, it is conceivable that the protection provided by exercise training might also result in protection against cell death. Thus, complementing the beneficial impact of PE on redox modulation and mitochondrial and ER function, along with its insulin-like activity and anti-inflammatory role, other positive effects may contribute to the valuable influence upon the apoptotic signaling [5, 97, 221, 222]. Most probably, this could occur by the reduction of the activities of JNK and caspases-8 and -9 rather than by the abrogation of growth arrest and DNA damage inducible protein (GADD34) and caspase-12 expression [4].

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AIMS

The purpose of this study was:

General purpose: To analyze the effects of life-long physical activity (LLPA) and ET on hepatic ER stress in obesity-induced NASH.

Study I: To analyze the preventive (voluntary physical activity, VPA) and therapeutic (endurance training, ET) effect of exercise programs on the anatomical and clinical profiles in an animal model of HFD-induced NASH.

Study II: To analyze the effects of VPA and ET upon hepatic insulin, pro-inflammatory and glucocorticoid signaling regulators/mediators in an animal model of HFD-induced NASH.

Study III: To analyze the effects of VPA and ET upon ER and oxidative stresses as well as ER-related pro-apoptotic signaling in an animal model of HFD-induced NASH.

CHAPTER 2: THEORETICAL BACKGROUND

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Endoplasmic Reticulum Stress Response in Non-alcoholic Steatohepatitis: The Possible Role of Physical Exercise

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ABSTRACT

Sedentary lifestyle coupled with excessive consumption of high caloric food has been related to the epidemic increase of non-alcoholic fatty liver disease, which can progress from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and, eventually, may culminate in hepatocellular carcinoma. Although the precise mechanisms underlying the progression of NASH are not completely understood, endoplasmic reticulum (ER) dysfunction seems to play a key role in the process. Hepatic ER stress has been associated to hepatic steatosis, insulin resistance, inflammation, oxidative stress and hepatocyte death, contributing to liver dysfunction. Physical exercise seems to be the most effective preventive and therapeutic non-pharmacological strategy to mitigate several features related to NASH, possibly targeting most of the referred mechanisms associated with the pathophysiology of ER-related NASH. Nevertheless, little is known about the impact of physical exercise on NASH-related ER stress. In this review, we will discuss the ER stress associated to NASH conditions and highlight the possible benefits of physical exercise in the attenuation and/or reversion of NASH-related ER stress.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; NASH, non-alcoholic steatohepatitis; ER, endoplasmic reticulum; ERAD, ER-associated degradation; UPR, unfolded protein response; PERK, protein kinase RNA-activated (PKR)-like ER kinase; IRE1, inositol requiring enzyme 1; ATF-6, activating transcription factor 6; BiP, binding immunoglobulin protein; eIF2α, eukaryotic translation initiation factor-2 α; ATF-4, activating transcription factor 4; ERO1, endoplasmic reticulum oxireductin 1; Nrf2, nuclear erythroid 2 p45-related factor; GADD34, growth arrest and DNA damage-inducible protein; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; S1P, site-1 protease; S2P, site-2 protease; XBP1, X-box binding protein-1; SREBP, sterol regulatory element-binding protein; SERCA, sarco/ER pump calcium ATPase; ROS, reactive oxygen species; IκB, inhibitor of NF-κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNFα, tumor-necrosis factor α; TRAF2, TNF-receptor-associated factor 2; CREBH, cyclic AMP response element-binding protein H; GRP94, glucose related protein 94; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; PI3K, phosphoinositide 3-kinase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol (3,4,5)-trisphosphate; ASK1, apoptosis signaling-regulating kinase 1; CHOP, C/EBP homologous protein; PTP1B, protein tyrosine phosphatase 1B; Keap1, Kelch ECH associating protein 1; PDI, protein disulfide isomerase; FAD, flavin adenine dinucleotide; MAM, mitochondrial-associated ER membrane; HFD, high-fat diet; TLR, Toll-like receptor; PBA, 4-phenyl butyric acid; TUDCA, taurine-conjugated ursodeoxycholic acid.

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1. Introduction

Sedentary lifestyle coupled with excessive consumption of high-caloric diets has been linked to the manifestation of metabolism-related pathological signs, including those characterizing non-alcoholic fatty liver disease (NAFLD) [1–3]. The relevant role of liver in systemic metabolic regulation argues for the association of NAFLD and metabolic dysfunction [3,4]. NAFLD represents the hepatic manifestation of the metabolic syndrome, which includes central obesity, hypertension, glucose intolerance and dyslipidemia, in addition to pro-inflammatory and pro-trombotic states, and comprises an increased risk for type 2 diabetes mellitus (T2DM) and atherosclerotic cardiovascular events [4–8]. In this scenario, elevated levels of circulating free fatty acids, and their consequent uptake by the liver, appears to play a major role in the development and progression of hepatic steatosis and insulin resistance [8–11]. In fact, aberrant hepatic lipid droplet accumulation has been associated with NAFLD that can progress from steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and, eventually, to hepatocellular carcinoma [2,8,12,13]. While hepatic steatosis, insulin resistance and oxidative stress characterize both NAFLD and NASH, NASH, in particular, is also associated with increased production of inflammatory mediators, increased apoptotic signaling and fibrosis in the liver [2,12]. Mitochondrial dysfunction seems to be involved in the onset and/or progression of NASH [14–16]. Additionally, prolonged endoplasmic reticulum (ER) dysfunction is also implicated in the regulation of liver lipid metabolism as well as in the development of steatosis and NASH [13,17–23].

ER has an important role as a sensor of cellular stress [24–26]. Under pathological and/or stressful conditions, in which the demand of protein synthesis is increased (e.g. NASH), an imbalance between the load of needed protein-folding and the response-related capability of the ER can lead to the accumulation of immature and/or misfolded proteins within the ER lumen [22,25,26]. This results in compromised functionality of this organelle and decreased mitochondrial function, ultimately leading to liver dysfunction [19,24]. Under both unmitigated ER and mitochondrial dysfunctions, restoration of ER homeostasis fails and the activation of the apoptotic signaling occurs [19,27]. Therefore, critical steps of ER dysfunction-related signaling pathways could be interesting therapeutic targets in NASH [28].

Several epidemiological and observational studies suggest that physical inactivity associates with obesity, inflammation, insulin resistance, T2DM and NASH [2,21,22,29]. On the other hand, interventional studies demonstrate that regular physical exercise has the potential to modulate several features related to the metabolic syndrome and NASH. In particular, it decreases both oxidative stress and inflammation, improves insulin sensitivity and reverses mitochondrial and ER dysfunctions [30–38].

In this review, we briefly discuss the ER dysfunction-induced NASH and the related inflammation, insulin resistance, oxidative stress and apoptotic signaling pathways. Thereafter, besides a brief presentation on exogenous chemical chaperones as relevant pharmacological interventions, we present and discuss detailed data that highlight the benefits of chronic regular physical exercise as a non-pharmacological therapeutic tool in the prevention, attenuation and/or reversion of NASH-related ER dysfunction and its associated deleterious consequences.

2. ER stress-induced unfolded protein response

Besides being a critical site for intracellular storage of calcium in the cell, the ER is also a vast dynamic and tubular network responsible for the synthesis, maturation, quality control and trafficking of a wide range of proteins. Therefore, the ability of the ER to adapt and manage adverse conditions is vital for the cell [21,26]. Only correctly folded proteins exit the ER to their final destination. So, unfolded and misfolded proteins are either retained in the ER lumen, with the molecular chaperones, to be correctly refolded or directed toward proteasomal degradation, in a process called ER-associated degradation (ERAD) [39]. Under pathological and/or stressful conditions, such as NASH, and due to the high demand for protein synthesis, folding and/or repair, decreased efficiency in the ER protein-folding, quality control and/or trafficking machinery is observed, most particularly in highly metabolic tissues (like the liver). This leads to a cellular perturbation termed *ER stress*, which results in the accumulation of unfolded and/or misfolded proteins [20–22,26,40]. In an attempt to restore homeostasis, ER elicits an elaborated adaptive response collectively known as the *unfolded protein response* (UPR) [26,40]. The UPR is characterized by the activation of three distinct signal transduction pathways mediated by three transmembrane sensors: protein kinase RNA-activated (PKR)-like ER kinase (PERK), inositol requiring enzyme (IRE) 1 and activating transcription factor (ATF)-6 [19,26]. In coordination, the three branches of the UPR regulate the transcription of various genes involved in the expansion of ER, the decrease of protein synthesis and the increase of protein folding capability and the degradation of terminally unfolded and misfolded proteins within ER [19,26,40].

In the absence of ER stress, the three transmembrane UPR sensors are maintained in an inactive state through binding to the ER chaperone binding immunoglobulin protein (BiP) [24]. However, when the protein load in the ER overcomes the ER folding or quality control capability, BiP detaches activating the UPR. This event results in the oligomerization and activation of both PERK and IRE1 through trans-autophosphorylation while ATF-6 translocates to the Golgi complex [26].

When activated, PERK phosphorylates the eukaryotic translation initiation factor-2 (eIF2)α, leading to immediate transient attenuation of protein synthesis, and, subsequently, prevents continued influx of newly synthesized polypeptides into the ER, thus reducing the ER workload [21,22,24,39]. However, considering that some level of protein-synthesis should be maintained, this pathway selectively induces the translation of ATF-4 and the expression of genes involved in ER redox control [endoplasmic reticulum oxireductin 1 (ERO1) and nuclear erythroid 2 p45-related factor (Nrf2)] [24,41,42]. Additionally, the PERK pathway is also responsible for cell recovery from protein synthesis shut-off through dephosphorylation of eIF2α [by growth arrest and DNA damage-inducible protein (GADD34)].

Moreover, activation of the PERK pathway also results in nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation, a master transcription factor relevant in the regulation of the inflammatory response, among other functions [24,25].

After translocation to the Golgi apparatus, ATF-6 is cleaved by site-1 and site-2 proteases (S1P and S2P) yielding its active form, which translocates to the nucleus and induces up-regulation of ER chaperones involved in protein folding [24,26].

Finally, the endoribonuclease activity of IRE1 removes a small intron from full-length (unspliced) X-box binding protein (XBP) 1 resulting in the generation of spliced XBP1 [21]. Spliced XBP1, alone or in coordination with ATF-6, initiates a transcriptional program in order to induce the expression of chaperones and molecules involved in ER biogenesis, phospholipid synthesis and ERAD [21,24].

All together, these three arms coordinately mitigate the ER stress induced by a high functional load by reducing global protein synthesis, increasing misfolded or unfolded protein degradation and, simultaneously, increasing the specific expression of proteins that help maintaining the protein folding process in the ER lumen as well as ER integrity [24,26,43]. Nevertheless, if these adaptive responses fail to attenuate the ER stress and ER homeostasis is not restored, cell function is compromised and apoptotic-signaling pathways may be triggered leading to cell death [21,22,40,43].

3. ER stress in non-alcoholic steatohepatitis (NASH)

In liver, ectopic fat accumulation coupled with disturbed fatty acid and cholesterol metabolism, increased oxidative stress, decreased mitochondrial function and mitochondrial ultrastructure disruption [14–16], insulin resistance [44], altered lipid composition [12,19,23], inflammation and apoptotic signaling [2,19] is related to the onset and progression of NASH. Moreover, although the precise mechanisms are not completely understood [22], adipose tissue dysfunction-induced deregulated adipokine production and increased release of free fatty acids, associated with excessive uptake by the liver, have been suggested to play an important role in the development and progression of NASH [8–11]. Recently, it has been proposed that ER stress within adipose tissue augments lipolysis and, consequently, could represent the underlying mechanism that contributes to increased circulating levels of free fatty acids and their increased uptake by the liver [45]. Hepatic ER stress seems to play an important role regulating the composition and size of lipid droplets as well as lipid synthesis, including cholesterol metabolism [3,13,22,24], through sterol regulatory element-binding proteins (SREBP). The SREBP protein family (from SREBP-1 and SREBP-2 genes in humans) is synthesized and located in the ER membrane, consisting its activation (similar to the ATF-6 activation process) in the yielding of the SREBP transcriptionally active forms: SREBP-1a, SREBP-1c and mature SREBP-2 [17,22,23,46]. Recently, it was demonstrated that ER stress in *ob/ob* mice promotes SREBP-1c activation and, thus, contributes to hepatic lipogenesis, while overexpression of the ER chaperone BiP decreases ER stress, and slows down lipogenesis, by inhibiting SREBP-1c activation and SREBP-1c target genes expression [17]. In response to tunicamycin, a pharmacological inducer of ER stress, and concomitantly of NASH, mice revealed a dramatic increase in hepatic active forms of SREBP-1a, SREBP-1c and NF-κB as well as a decrease in the expression of genes involved in lipolysis and fatty acid oxidation [23]. In line, XBP1 deficiency reduced the serum levels of triglycerides and cholesterol and induced resistance to liver steatosis development under a carbohydrate-rich diet [47]. Additionally, the IRE1 pathway regulates the transcription of several genes involved in fatty acid and triacylglycerides synthesis, such as stearoyl-CoA desaturase-1, acetyl-CoA carboxylase 2 and diacyl-glycerol-acyl transferase 2 [24]. However, it is noteworthy that increased hepatic accumulation of saturated fatty acids [48,49] and inflammatory cytokines, typical NASH-related features, can also activate the UPR [25,50], suggesting that the relationship between ER stress and lipid metabolism is bidirectional. Circulating free fatty acids, which are increased in NASH patients and linked to the severity of the disease [9], can induce both hepatic and adipose tissue ER stress, being saturated fatty acids more cytotoxic than unsaturated fatty acids [3,10,49]. In fact, palmitic acid can induce apoptosis, accompanied by autophagy, through ER stress and mitochondrial dysfunction [49], while oleic acid can revert palmitic acid -induced lipotoxicity [51]. Sarco/ER pump calcium ATPase (SERCA) functionality disruption can occur after an increase in the saturation level of the ER membrane [9]. Furthermore, alteration in ER phospholipid composition (higher concentration of phosphatidylcholine than phosphatidylethanolamine), in parallel with increased stimulation of lipid synthesis observed in the obese liver [3,18], disrupts calcium homeostasis and induces ER stress, via inhibition of SERCA2b activity [18]. On the other hand, hepatic enhanced or restored SERCA2b activity mitigates hepatic ER stress and reactive oxygen species (ROS) production, decreases hepatic lipogenesis and triglyceride accumulation, and protects the hepatocyte from ER stress-induced apoptosis, and so decreasing the risk for NASH onset and progression [21,22,46,52].

In this regard, we can argue that reducing adipose tissue mass (and consequently, free fatty acids release) and inflammatory signaling in visceral adipose tissue and liver as well as increasing adipose and hepatic ER and mitochondrial functionality could be of paramount importance to the favorable management of the metabolic syndrome and, consequently, of NAFLD/NASH. Interventional strategies, including physical exercise, could be used to achieve this purpose.

Considering the relevance of inflammation, insulin resistance, oxidative stress and apoptotic signaling, a detailed molecular mechanistic insight into the role of these crucial key players in NASH-related ER stress will be addressed in the following sections.

3.1. *ER stress induces inflammation in NASH*

ER stress has been suggested to be a key mediator of hepatic inflammation in the context of NASH. In line, UPR contributes to the inflammatory response associated with NASH, through phosphorylation of the inhibitor of NF-κB (IκB) by IkB kinase (IKK) (NF-κB is maintained in the quiescent state through binding to IκB and IκB phosphorylation leads to its ubiquination and proteosomal degradation leading to NF-κB activation) as well as through the activation of c-Jun N-terminal kinase (JNK)/activator protein 1 (AP1) [19,21–25,50,53].

It is well recognized that PERK and IRE1 pathways play important roles in the interconnection of ER stress with the inflammatory signaling pathway [25,54–57]. PERK-mediated translational repression of protein translation leads to decreased IκB expression and, consequently, to NF-κB activation [25,57]. When activated, NF-κB can induce or exacerbate the inflammatory signaling, prompting the expression of interleukin (IL)-1β, IL-6 and tumor-necrosis factor (TNF) α [25,56,58,59]. IRE1 pathway can activate both JNK and IKK, through its kinase domain and interaction with TNF-receptor-associated factor 2 (TRAF2), leading to increased expression of pro-inflammatory mediators, such as TNFα, IL-1β and IL-6, as well as to NF-κB activation [25,54,55]. In turn, TNFα binding to its receptor can lead to IKK and JNK activation [60–62]. Also, IL-1β, IL-6 and TNFα induce the activation of cyclic AMP response element-binding protein H (CREBH). CREBH is an ER stress-associated liver-specific transcription factor whose activation requires its translocation to the Golgi compartment and cleavage by S1P and S2P, as occurs with ATF-6 and SREBP. Even though ER stress-induced cleavage of CREBH does not lead to increased transcription of UPR genes, mature CREBH interacts with ATF-6 and, synergistically, induces transcription of many acute-phase response genes in the liver, such as C-reactive protein and serum amyloid P-component, contributing to and worsening the NASH-related systemic inflammatory response [22,25,50].

In addition, exacerbated oxidative stress can induce JNK activation leading to the increment of pro-inflammatory factors, such as TNFα and IL-6 [25,39]. On the other hand, pro-inflammatory factors, such as IL-1β, TNFα and interferon-γ, are able to induce ER stress, to decrease SERCA2b activity and, eventually, to downregulate the expression of several ER chaperones, such as BiP and glucose related protein 94 (GRP94) [63,64]. Therefore, ER stress can amplify the inflammatory signaling, even in conditions of mild ER stress [65], but inflammation can also induce ER stress, thereby generating a vicious cycle. ER stress signaling, which is instrumental in the development of NASH, cooperates synergistically with pro-inflammatory signaling pathways through TNFα and IKKβ signaling to promote hepatocarcinogenesis [13,66]. As we will discuss below, the anti-inflammatory feature of physical exercise seems to positively contribute to mitigate this ER stress-related inflammatory response.

3.2. *ER stress contributes to insulin resistance in NASH*

The precise mechanisms by which ER stress decreases insulin signaling and metabolic control are complex (involving activation of PERK and/or IRE1 pathways) and may occur at several and distinct levels of the insulin signaling cascade [20,21,25,43].

Insulin initiates its pleiotropic effects through sequential induction of insulin receptor and insulin receptor substrates (IRS) phosphorylation. IRS1 and IRS2 (the most relevant IRS for insulin metabolic effects in the liver) phosphorylation on specific stimulatory tyrosine residues (insulin receptor mediated, after its own activation by autocatalytic stimulatory tyrosine residues phosphorylation) stimulates the enzyme phosphoinositide 3-kinase (PI3K). PI3K conversion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) into phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3) leads to protein kinase B (also known as Akt) activation, one major branch point in the insulin signaling cascade [61,67,68]. In contrast, IRS1 and IRS2 phosphorylation on inhibitory serine residues prevents the insulin signaling cascade, contributing to insulin resistance development, and is fundamental in the control of the insulin signaling pathway, under both physiological and pathological conditions [17,68–70].

Under NASH-associated ER stress, IRE1 recruits TRAF2 and apoptotic signaling-regulating kinase 1 (ASK1) to activate JNK and IKK, which are critically involved in the promotion of insulin resistance [56,68,71]. In fact, JNK activation, particularly isoform 1 [19,56,71], mediates the phosphorylation of inhibitory serine residues of both IRS1 and IRS2 [19,56,71], contributing to insulin resistance. Accordingly, ablation of JNK1 [71,72] or mutation of serine^{307} to alanine^{307} substantially decreases the development of insulin resistance [60]. On the other hand, IKK can also disrupt the insulin signaling pathway through the inhibitory serine residues phosphorylation of IRS1 and IRS2 [70].

Another mechanism through which JNK1, IKK and NF-κB can induce insulin resistance is by induction of pro-inflammatory cytokines [56,58,59]. In fact, inflammation might be one of the most important factors that triggers insulin resistance in the context of NASH [73], being C/EBP homologous protein (CHOP) a key player in this process [74]. Mice with CHOP deletion, despite presenting hepatic steatosis, showed preserved normal hepatic insulin sensitivity accompanied by lower hepatic inflammation [74].

Additionally, protein kinase C activity has also been implicated in the down-regulation of insulin signaling by direct phosphorylation of IRS1 or insulin receptor on inhibitory serine residues or by up-regulation of inflammatory signaling [75].

Several studies suggest that protein tyrosine phosphatase 1B (PTP1B), which is anchored in the cytosolic side of the ER, could be one of the major negative regulators of insulin sensitivity through tyrosine residues dephosphorylation of insulin receptor, IRS1 and IRS2 [70,76–78]. Accordingly, ablation of PTP1B in mice results in increased systemic insulin sensitivity and improved glucose tolerance and lipid profile [76,78], therefore establishing PTP1B as an important target of treatment against NASH-related insulin resistance. PTP1B can be induced by ER stress via the activation of the ROS-NFκB axis [79].

In this regard, we can argue that reducing the hepatic inflammatory signaling, as well as visceral adipose tissue mass and inflammatory signaling, seems to be important to favorably manage the metabolic syndrome and, consequently, NAFLD/NASH. As will be discussed below in detail, physical exercise has been suggested as a useful and efficient strategy to mitigate insulin resistance in NASH.

3.3. *ER stress induces oxidative stress in NASH*

Oxidative stress results from an imbalance between ROS generation and ROS detoxification [41,80], being mitochondria not only important sources of intracellular ROS production but also major targets of ROS-induced damage predisposing them to dysfunction [81]. Concomitantly, the decline in mitochondrial functionality may lead to enhanced ROS generation, which further increases mitochondrial dysfunction [15,39,82].

In the context of NASH, nutrient overload-induced excessive mitochondrial metabolic oxidation and concomitant exacerbated

mitochondrial ROS production have been described [15]. Moreover, as detailed below, this mitochondrial dysfunction-induced increased ROS production promotes an ER stress response that, in turn, exacerbates mitochondrial ROS production and dysfunction through a vicious cycle mechanism [24,82,83].

As a first line of response during UPR activation, ER-related PERK/eIF2-α pathway not only attenuates general mRNA translation but also, specifically, activates the Nrf2 transcription factor, a key player in antioxidant response and intermediary metabolism regulation [24,41,42,80,84]. Under normal physiological conditions, Nrf2 signaling is repressed by its interaction with Kelch ECH associating protein 1 (Keap1). However, under pro-oxidant conditions Nrf2 is released from Keap1, translocates to the nucleus and activates antioxidant responsive element-dependent gene expression [24–26,41,80]. Nevertheless, under pathological conditions, such as NASH or cirrhosis, the UPR-induced Nrf2-mediated response is down-regulated [85]. Impaired Nrf2 activity associates with mitochondrial depolarization/dysfunction, as well as increased hepatic free fatty acid levels, fatty liver and NASH development [84,86,87].

NASH-related excessive accumulation of misfolded proteins, and related unmitigated ER stress, also induces increased ROS production and macromolecules oxidation in the ER lumen resulting in intracellular depletion of reduced glutathione with concomitant increase of oxidized glutathione [39,80,88]. In fact, although ROS are produced in all cellular compartments, it has been estimated that the oxidative protein folding process may be responsible for approximately 25% of total cellular ROS production [89]. Therefore, ER has also been suggested as a major site of ROS production [90]. Under ER stress conditions, emerging evidence suggests that protein disulfide isomerase (PDI), which catalyzes disulfide bond formation and isomerisation, is involved in increased ROS generation. When oxidized, PDI acts in the oxidative folding of reduced proteins, by accepting hydrogen atoms and allowing disulfide bond formation in the target proteins. ERO1 binds and reacts with reduced PDI, oxidizing PDI thiols to disulfides. ERO1 transfers hydrogen atoms from PDI to its cofactor flavin adenine dinucleotide (FAD) and, then, hydrogen atoms flow from $FADH_2$ to molecular oxygen producing hydrogen peroxide as a by-product. So, ERO1 and PDI may re-enter into another cycle of substrate disulfide formation. When reduced, PDI breaks and rearranges disulfides in the nascent proteins until the reduced glutathione pool is depleted [80,90]. In addition, ROS may also be formed as a result of glutathione depletion, the main redox buffer in the ER lumen, as glutathione reduces unstable and improper protein disulfide bonds [80,88]. Furthermore, both ER stress and oxidative damage prompt calcium leak from the ER, leading to mitochondrial calcium accumulation, which in turn promotes exacerbated mitochondrial ROS production, further amplifying ER stress [39,82,88]. However, it has been recently suggested that elevated levels of palmitic acid would compromise the ER capability to maintain calcium stores, resulting in the stimulation of mitochondrial oxidative metabolism, ROS production and, ultimately, cellular dysfunction [9]. Therefore, it appears that ER stress may occur earliest than the onset of mitochondrial dysfunction, ROS accumulation and apoptosis [9,51]. Nevertheless, the reinforced cycle of ER stress, oxidative stress and lipogenesis-induced lipotoxicity aggravates NASH [13].

Given the known positive effect of physical exercise in the modulation of cell antioxidant defense systems, as well as its possible role on the improvement of the ER stress response, one should consider physical exercise as an effective non-pharmacological tool to mitigate (both preventing and/or treating) this harmful vicious cycle.

3.4. *ER stress promotes cell-death in NASH*

Despite having a rather robust and elegant adaptive system, the UPR does not always successfully alleviate ER stress and restore ER homeostasis [43,91]. In fact, it has been suggested that depending on the nature and severity of the ER stress, the UPR acts as a binary switch, regulating adaptive and death effectors, thus controlling cellular life and death decisions [40,91]. Under sustained and unmitigated ER stress conditions, such as those observed in NASH, apoptotic signaling is generated [21,22,40,92] through several mechanisms, including: (I) PERK/eIF2α-dependent induction of the pro-apoptotic transcription factor CHOP; (II) IRE1-mediated activation of apoptotic signaling by ASK1/JNK; (III) cleavage and activation of pro-caspase-12, mediated by IRE1 binding to TRAF2; and (IV) release of calcium from the ER [21,22,40,55,92].

Regarding PERK/eIF2α/CHOP and IRE1/ASK/JNK pathways, both induce apoptosis by decreasing anti-apoptotic protein Bcl2 and/or by increasing pro-apoptotic proteins BIM and Bax activity/expression [82,88]. Through CHOP activation, the PERK/eIF2α pathway also increases GADD34 expression that is responsible for shutting down the general translational repression, previously mediated by PERK/eIF2α pathway, through eIF2α dephosphorylation [93]. Therefore, the increment of newly synthesized proteins in the already stressed ER milieu results in an additional cargo to the ER and, consequently, in UPR chronic activation, which reinforces the activation of cell-death pathways [39]. On the other hand, the binding of IRE1 to TRAF2 can also induce apoptotic signaling through the activation of the ER membrane resident caspase-12 [94], which directly activates caspase-9 independently of the mitochondrial release of cytochrome *c* (even though mitochondria might also be involved in the amplification of ER stress-induced apoptosis) [92].

Increasing evidence highlights the functional relevance of the mitochondria-ER cross-talk during the UPR. In fact, structural and functional interaction between ER and mitochondrial network, i.e. interorganellar bridges called mitochondrial-associated ER membrane (MAM), may provide a selective direct pathway for calcium movement from the ER to mitochondria [27,95].

The ER stress-induced ROS production may cause calcium leak from the ER, leading to mitochondrial calcium accumulation, mitochondrial depolarization/dysfunction and increased mitochondrial ROS production, which further amplifies the ER stress [9,82,88]. In UPR early phases, calcium, a key regulator of mitochondrial function, stimulates mitochondrial respiration [96]; however, under sustained ER stress conditions, ERO1-mediates massive calcium release through MAM [95], due to decreased SERCA2b activity, leading to the formation of calcium microdomains along MAM that are buffered by mitochondria [18,46]. As a consequence, mitochondria swell, membrane potential collapses and mitochondria outer membrane disrupts releasing cytochrome *c* [27]. This reinforces the key role of ER in

the regulation of the mitochondrial intrinsic apoptotic pathway by adjusting the load of calcium imposed upon mitochondria [9,27,40]. As previously mentioned, here we hypothesize that physical exercise can positively interfere with this vicious cycle, increasing the functionality of the ER as well as enhancing the capability of mitochondria to buffer calcium and, consequently, blocking the propagation of the apoptotic signaling.

4. Treatment strategies targeting ER stress-related components of NASH

The imbalance between excessive caloric intake and low energy expenditure promotes obesity, which is closely related to several leading causes of death, including metabolic syndrome, T2DM, NASH, cardiovascular disease and cancer. Therefore, identifying potential preventive and therapeutic strategies to counteract this cluster of diseases has been considered a primary health-related goal.

No approved pharmacological therapies for the treatment of NAFLD/NASH have yet been identified. The increasing understanding of the molecular mechanisms underlying hepatic ER stress in the onset and/or progression of NAFLD/NASH led to the development of pharmacological strategies in order to counteract this ER dysfunction-related disorder [7,9,20,22,28,31,48,97–108]. However, it should be emphasized that besides genetic predisposition, sedentary lifestyle and high-caloric food consumption represent the central elements responsible for the onset and progression of NASH [2]. Therefore, weight loss [109] and physical exercise [31,100,110], besides chemical chaperones [7,28,98,99,101–103,107,108,111], might play an important role in the attenuation of ER stress in several tissues.

4.1. *Physical exercise as a non-pharmacological therapeutic strategy*

Despite the precise molecular mechanisms underlying the beneficial effects of physical exercise against some pathological conditions (e.g. obesity, insulin resistance, NAFLD/NASH, diabetes, heart failure and cancer) are not completely elucidated [36,104–106,112], different studies suggest that positive modulatory effects occur at distinct levels of cellular organization [113].

4.1.1. *The molecular-level effects of physical exercise*

It seems clear that physical exercise is able to mitigate several features related to NASH, including the reduction of hepatic fat and visceral adiposity, reduction of chronic low-grade inflammation and oxidative stress, improvement of insulin signaling and mitochondrial function, and attenuation of apoptotic signaling [29,30,33,34,37,114,115].

4.1.1.1. *The molecular-level effects of physical exercise: on redox state.* A variety of mechanisms could be associated with the protective effects of physical exercise in several tissues, such as cardiac, renal and hepatic, including redox changes and improvement of antioxidant capacity, as described by several researchers including us [7,37,113]. The boosting of the physical exercise-induced intrinsic antioxidant potential is, most possibly, mediated by Nrf2 [116,117].

4.1.1.2. *The molecular-level effects of physical exercise: on inflammation.* The inflammatory pathway could also be a potential target for NASH prevention and treatment through lifestyle interventions, particularly exercise [114,115]. In fact, several studies demonstrated that physical exercise reverses the mRNA expression pattern of inflammatory cytokines, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1 and IKKβ in the adipose tissue of an animal model of high-fat diet (HFD) [30,34]. Moreover, the exercise-decreased circulating levels and adipose, pancreatic and hepatic mRNA and/or protein expression of TNF-α and IL-6, and of Toll-like receptor (TLR) 4 hepatic, adipose and muscle mRNA and protein expression, reinforce that physical exercise is also anti-inflammatory in nature in the context of metabolic diseases [34,35,118,119]. In accordance, physical exercise prevented the increase in the protein expression of NF-κB and phosphorylation levels of JNK and IκB in the liver and adipose tissue [32] of rats submitted to HFD-induced obesity.

4.1.1.3. *The molecular-level effects of physical exercise: on insulin resistance.* Regarding insulin resistance, it is known that physical exercise improved insulin sensitivity in animal models of diet-induced obesity [30,32,35] and reduced hepatic fat and visceral adipose tissue and insulin resistance in obese adolescents [120]. In addition, increased phosphorylation of the insulin receptor, IRS1 and Akt [32] and reduced inhibitory phosphorylation of IRS1 on serine^{307} as well as improved insulin-induced insulin receptor and IRS1 tyrosine residues phosphorylation and Akt serine residues phosphorylation [35] were observed in diet-induced obese rats after exercise training.

4.1.1.4. *The molecular-level effects of physical exercise: on apoptotic signaling.* Complementing the impact of physical exercise on redox modulation, anti-inflammatory function, and insulin-like activity, other beneficial effects seem to be related to its favorable influence on apoptotic signaling. Thyfault et al. [121] reported that rats selectively bred for high-aerobic capacity have reduced susceptibility to steatosis development, progression and apoptotic signaling. Accordingly, Fealy et al. [122] showed that short-term exercise decreased circulatory markers of hepatocyte apoptosis in NAFLD patients. Recent data from our group also showed that endurance training was able to positively counteract deleterious hepatic histological alterations as well as mitochondrial unfitness characterizing HFD-induced NASH [36,104].

4.1.1.5. *The molecular-level effects of physical exercise: on ER stress.* Studies analyzing the effect of chronic physical exercise on ER stress response in other tissues rather than in the liver have also been conducted. Interesting, high-voluntary running activity was associated with increased UPR in the hypothalamus (XBP1, ATF-6, eIF2α and BiP), hippocampus (XBP1, ATF-6, eIF2α and BiP) and cortex (ATF-6 and eIF2α) [110]. However, decreased PERK and eIF2α phosphorylation in adipose tissue of diet-induced obesity was observed after a chronic swimming exercise protocol [32]. These apparent contradictory results (increased UPR vs. decreased UPR) suggest that the effects of physical exercise in ER stress are tissue-specific and might function as a binary switch, increasing the protective and pro-survival response and

repressing death effectors. Nevertheless, the tipping points that decide the increasing vs. decreasing UPR response perpetrated by physical exercise still remain obscure requiring further investigation. We postulate that the direction of this binary switch depends on the tissue, the nature and/or intensity of the ER stress, the phase of UPR response and the expression or activation of survival and/or death signaling components.

Additionally, it has been suggested that UPR is activated and contributes to skeletal muscle adaptations to exercise [100]. Peroxisome proliferator-activated receptor-γ coactivator-1α seems to act as an important transcriptional co-regulator of the UPR in skeletal muscle during physical exercise through the co-activation of ATF-6 [100]. In this regard, the attenuated expression of some molecular markers closely associated to the activation of cell-death mechanisms through exercise training in the skeletal muscle, including ATF-4 and CHOP, seems to reinforce the role of physical exercise in the improvement of ER fitness/resistance [100]. In an attempt to better analyze the mechanisms by which ER stress mediates insulin resistance in skeletal muscle, Yang et al. [123] showed that HFD decreased the expression of IL-15, a cytokine associated with improved insulin sensitivity, and that endurance training mitigated this effect. After *in vitro* pre-incubation of muscle strips with an ER stress inducer, IL-15 treatment decreased the protein expression of several markers of ER stress (BiP, GRP94 and CHOP), possibly explaining the improved insulin sensitivity induced by physical exercise [123].

It is plausible that physical exercise may be an effective non-pharmacological preventive and therapeutic strategy against diet-induced ER stress in NASH-inducing conditions. However, only few studies showed its putative positive effects on NASH-related ER stress.

The beneficial effects of physical exercise seem to be associated to its capability to increase the expression of the adaptive UPR-related genes and/or to decrease the expression of pro-apoptotic-related genes. Indeed, high-voluntary running activity was associated with increased mRNA expression of ATF-6 and eIF2α in the liver of HFD-fed mice [110]. Nevertheless, no effects were observed in the mRNA expression of XBP1 and BiP [110], while the expression of factors related to ER stress-induced activation of apoptotic signaling was not analyzed. Additionally, forced exercise training resulted in increased mRNA and/or protein expression of BiP, ATF-6, PERK, p-PERK, PDI, IRE-1 and XBP1 in the liver in a model of HFD-fed rodents [31,124]. Also, while exercise training *per se* was not able to modulate the hepatic expression of CHOP and ATF-4 in mice submitted to HFD [124], da Luz et al. [32] showed that the increased rat hepatic protein expression of p-PERK and eIF2α in HFD-induced obesity was mitigated after a swimming training program.

The functionality of the ER protein processing machinery can be improved either by enhancing the protein folding and transport of nascent proteins or by enhancing the ER capability to remove misfolded proteins through the ERAD system [125]. In line with this, it would be interesting to understand the role of physical exercise on the dynamic modulation of the ERAD system in order to maintain homeostasis and protect against ER stress-related NASH.

Altogether, as no alterations induced by physical exercise were found in the hepatic expression of downstream ER stress-associated pro-apoptotic signaling markers, one can speculate that the above referred improvements in UPR could be considered beneficial adaptive mechanisms [31,32,124]. Consequently, hepatic tissue from exercised individuals has an increased protein folding capability, elevated ER expansion and decreased load of protein synthesis [19,22,26,40]. To adapt to metabolic stress, the downstream signaling output of the UPR must enhance the capability of the ER to process the new stressor client proteins to an extent enough to mitigate the perturbing effects of the stressor and, consequently, attenuate the UPR signaling. In other words, physical exercise probably stimulates ER to achieve a new state of equilibrium in which the UPR signaling maintains an improved functionality of the ER protein processing machinery, without induction of the deleterious effects of the sustained UPR activation and consequent activation of the apoptotic signaling cascade.

Studies exploring the role of physical exercise on the modulation of UPR in the context of NASH are yet scarce and remain inconclusive, being the inconsistent results possibly explained by the distinct animal models, strains and interventions, such as exercise type (swimming vs. treadmill), duration and/or intensity (voluntary physical activity vs. endurance training) used in the different protocols.

Generically, although some of the molecular signaling markers of UPR appear to present distinct tendencies or patterns in response to physical exercise, benefits to hepatic ER function and related physiological phenomena, including insulin sensitivity, could be anticipated. Moreover, even though physical exercise has been suggested as a protective stimulus against ER stress-induced up-regulation of pro-apoptotic-associated genes [110,124], it is not clear whether similar effects occur in all tissues.

Nevertheless, several questions arise. To what extent do the effects of physical exercise increase markers of UPR rather than reduce them? How does physical exercise dynamically induce increases in some downstream pathways and selectively reduce other ER stress markers? Thus, although the impact of physical exercise is an important and expanding research area [112], more studies are needed to better understand the molecular mechanisms underlying the beneficial effects of physical exercise against ER stress in the setting of several metabolic diseases, including NASH.

4.2. *Exogenous chemical chaperones as a pharmacological therapeutic strategy*

Besides lifestyle interventions, pharmacologic manipulation of the ER stress response could also protect cells from irreversible damage and offer novel opportunities for treating the cluster of metabolic diseases [20,111].

Exogenous chemical chaperones [which represent a group of low-molecular-weight compounds comprising, among others, betaine, 4-phenyl butyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA)] have been used to mimic endogenous chaperones and enhance the adaptive capability of the ER, probably through the stabilization of protein conformation, the improvement of ER folding capacity, the reduction of accumulation and aggregation of misfolded proteins in the ER lumen and the facilitation of mutant proteins trafficking [101,108,111].

Betaine has been reported to improve adipose tissue function and, consequently, to protect liver from NAFLD in

HFD-fed mice [7]. Betaine reduced fat accumulation and injury in liver, enhanced insulin sensitivity and attenuated ER stress response in adipose tissue (the latter by reducing JNK activation and GRP78 and CHOP protein expression), as well as corrected abnormal adipokine production profile [7].

Treatment of *ob/ob* mice (obese and diabetic mice) with PBA and TUDCA alleviated ER stress (by reducing PERK and IRE1 phosphorylation), which associated with the reversion of fatty liver disease, reestablishment of systemic insulin sensitivity, regularization of hyperglycemia and improvement of insulin action in liver, muscle and adipose tissue [111]. TUDCA counteracted several ER stress markers induced by palmitic acid, including phosphorylation of eIF2α, splicing of XBP1 and expression of BiP and ATF-4, in the liver of mice fed with a methionine-choline-deficient diet that induced NASH [99]. Additionally, the ability of TUDCA to protect cells against ER stress was also manifested in the blockage of apoptotic signaling activation. TUDCA has been shown to inhibit ER stress-induced apoptosis through inhibition of caspase-12 activation, blockage of JNK phosphorylation and decrease of CHOP up-regulation as well as through inhibition of calcium-mediated activation of apoptotic signaling [99,101,107]. Concomitantly, TUDCA hampers the mitochondrial apoptotic pathway by preventing the activation of Bax and the release of cytochrome c, by stabilizing the structure of proteins and lipids within the mitochondrial outer membrane as well as by decreasing oxidative stress [99,101,107]. In a randomized controlled trial in obese and insulin resistance individuals, TUDCA improved hepatic insulin signaling and glucose homeostasis [103].

However, although synergy of the beneficial effects could underlie the putative role of TUDCA in the attenuation and progression of NASH, the precise mechanisms of the well-demonstrated beneficial effects of exogenous chemical chaperones remain to be fully understood [101,108].

Overall, evidence strongly supports the feasibility of targeting ER stress, and its downstream pathways, as therapeutic goals in the context of metabolic disturbance, specifically NASH, both in humans and rodents models.

Nevertheless, it is important to point out that there is not yet an approved, efficient and safe pharmacological therapy to counteract the increasing prevalence of NASH. Therefore, besides utilization of chemical chaperones to decrease hepatic ER stress in the context of NASH, physical exercise may provide a balanced non-pharmacological therapeutic strategy for ER stress-related NASH.

5. The simultaneous modulation of cardiovascular disease risk factors and NAFLD by physical exercise

Nowadays, there is convincing evidence that the metabolic syndrome and its hepatic manifestation, NAFLD/NASH, associate with cardiovascular disease [8,61]. Concomitantly, several components of the metabolic syndrome, such as visceral obesity and insulin resistance, are considered to be independent risk factors for cardiovascular disease [61]. Additionally, sedentarism is considered a trigger factor of cardiovascular disease [34].

Considering NAFLD/NASH as a lifestyle-related disease, physical exercise is likely to be the primer line and hopeful option to both preventive and therapeutic measures against NAFLD/NASH [10,104].

The ability of physical exercise to reduce and/or improve metabolic syndrome features strongly argues that exercise-induced protection against cardiovascular disease is somewhat expectable. Physical exercise reduces waist circumference, visceral abdominal fat, blood pressure, insulin resistance and hepatic fat content in obese patients, while increasing cardiovascular fitness [120,126]. Adding to this, epidemiological studies have consistently documented a reduced incidence of coronary artery disease events in more physically active subjects, being physical activity considered both a preventive and therapeutic measure in many established atherosclerotic risk factors, including obesity, insulin resistance, glucose intolerance, elevated blood pressure, high triglyceride concentrations and low high-density lipoprotein cholesterol concentrations [127,128]. Likewise, in animal models, physical exercise reduced body weight and adiposity and improved lipid profile and insulin signaling as well as suppressed inflammatory signaling in the liver, muscle and adipose tissue [30,34,35]. Very recently, our group suggested physical exercise as a promising non-pharmacological strategy to counteract both anatomical features of obesity and histological hepatic features of NASH in HFD-induced obesity in rats [104].

6. Weaknesses and strengths

This review presents some limitations. First, it is based on studies with different methodologies and models (including human, rodent and *in vitro* studies). Although animal models of NAFLD/NASH share some mechanisms, the translation of results to humans must be interpreted with caution. Second, data regarding the effects of physical exercise on hepatic ER stress in the context of NASH are scarce and inconclusive. Third, whether physical exercise interferes with the ERAD system is yet unclear. Finally, the effect of physical exercise on ER stress-induced cell death is very limited and suggestions of how physical exercise could block the activation of cell death induced by ER stress have been anticipated. Despite these limitations, this review presents a comprehensive understanding of how physical exercise could dynamically manipulate the hepatic UPR signaling. Ultimately, it is proposed that physical exercise should be seen as a dynamic tool, able to switch from increasing ER stress markers to reducing ER stress markers.

7. Conclusion

NASH is one of the most common disorders in developed countries. Even though numerous pharmacological therapeutic approaches have been used to counteract this emerging pathology, physical exercise is considered a beneficial non-pharmacological preventive and therapeutic strategy against NASH-related physiopathology [29].

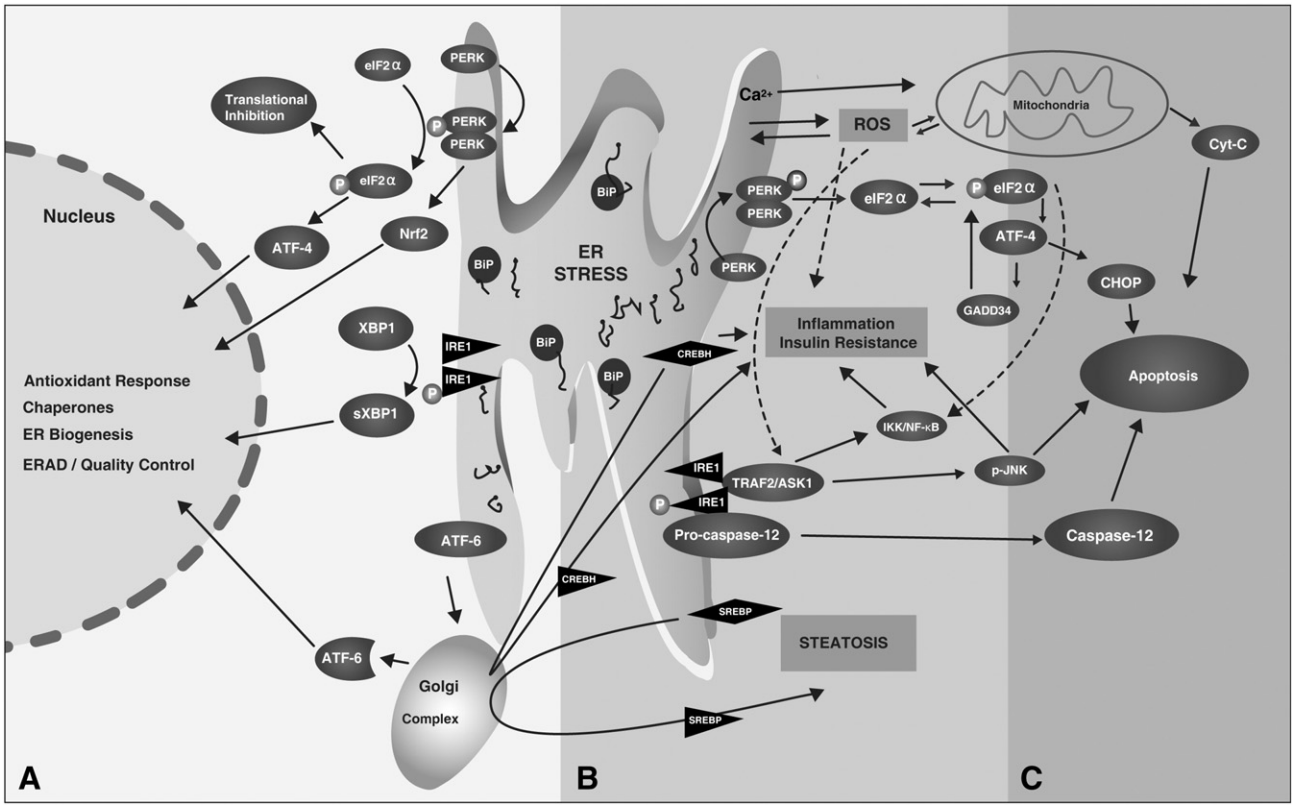

Fig. 1 – The activation of unfolded protein response (UPR). Panel A represents the first phase (the adaptive response) in which the three UPR sensors (PERK, IRE1, ATF6) are activated by BiP release, in an attempt to restore homeostasis. Panel B represents the alarm phase in which hepatic sustained UPR might result in ectopic fat deposition, inflammation and insulin resistance in the liver. Panel C represents the third phase in which ER stress is perpetuated, cells fail to restore ER homeostasis and apoptotic signaling pathways are triggered leading to hepatic cell-death.

Considering that an imperative inter-connection exists between the ER stress, ROS generation, chronic low-grade inflammation and apoptosis in the setting of metabolic diseases, including NASH, an integrative view regarding the widely known benefits of physical exercise must be taking into account. However, despite physical exercise-induced

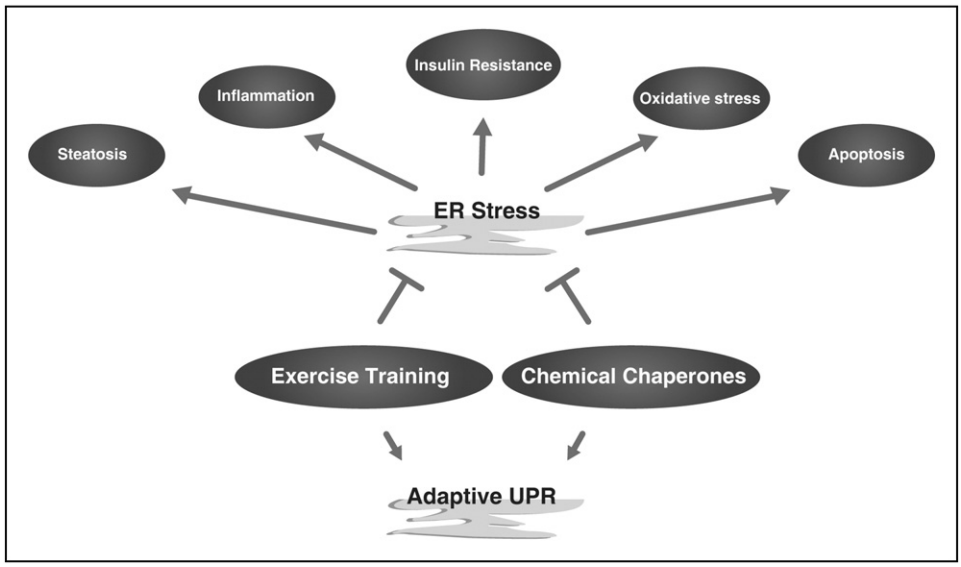

Fig. 2 – Hypothetical effects of chemical chaperones and physical exercise. Chemical chaperones and chronic physical exercise may prevent and/or revert ER stress, that in turn may have beneficial effects in several metabolic alterations.

prevention or attenuation of ER stress in the context of NASH is a promising area, only few studies have already examined its influence. Therefore, to reinforce the hypothesis that physical exercise could be a trusty factor that protects cells against NASH-induced ER stress by increasing the adaptive capacity of the ER to deal with unfolded or misfolded proteins, suppressing chronic low grade inflammation, controlling calcium signaling, restoring redox status and decreasing the expression of pro-apoptotic-related genes, future studies are needed to better understand the molecular mechanisms involved in this putative protective phenotype.

Author contributions

All the authors contributed to the design, preparation and writing of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 3: EXPERIMENTAL WORK

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Original article

Physical exercise antagonizes clinical and anatomical features characterizing Lieber-DeCarli diet-induced obesity and related metabolic disorders

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SUMMARY

Background & aims: Lieber-DeCarli diet has been used to induce obesity and non-alcoholic steatohepatitis (NASH). As scarce anatomical and clinical-related information on this diet model exists and being exercise an advised strategy to counteract metabolic diseases, we aimed to analyze the preventive (voluntary physical activity – VPA) and therapeutic (endurance training – ET) effect of exercise on clinical/anatomical features of rats fed with Lieber-DeCarli diet.

Methods: In the beginning of the protocol, Sprague-Dawley rats were divided into standard-diet sedentary (SS, $n = 20$), standard-diet VPA (SVPA, $n = 10$), high-fat diet sedentary (HS, $n = 20$) and high-fat diet VPA (HVPA, $n = 10$) groups. After 9-weeks, half ($n = 10$) of SS and HS groups were engaged in an ET program (8 wks/5 d/wk/60 min/day). At this time, a blood sample was collected for biochemical analysis. At the end of protocol (17-weeks) anatomic measures were assessed. Heart, liver, femur and visceral fat were weighted and blood was collected again. Liver section was used for histopathological examination.

Results: At 17-weeks, high-fat diet increased visceral adiposity (HS vs. SS), which was counteracted by both exercises. However, ET was the only intervention able to diminished obesity-related measures and the histological features of NASH. Moreover, blood analysis at 9 weeks showed that high-fat diet increased ALT, AST, cholesterol and HDL while VLDL and TG levels were decreased (HS vs. SS). Notably, although these parameters were counteracted after 9-weeks of VPA, they were transitory and not observed after 17-weeks.

Conclusions: ET used as a therapeutic tool mitigated the clinical/anatomical-related features induced by Liber-DeCarli diet, thus possibly contributing to control obesity and metabolic disorders.

1. Introduction

Obesity has been considered an increasing global epidemic health problem. Among several serious consequences, obesity is associated with numerous chronic diseases in the spectrum of the so-called metabolic syndrome, including its “hepatic expression” – the liver steatosis (non-alcoholic fatty liver disease (NAFLD). Although some genetic predispositions, together with increasing age, contributes to the development of obesity, “obesogenic environments”[1] that are nurturing over-eating and sedentary behaviors play a decisive role. Given the tremendous health, economic and social impact of obesity, several preventive and therapeutic approaches have been tested worldwide. However, in combination with healthy nutritional habits, one of the most effective strategies

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against this vicious scenario of inactivity and hypercaloric diet is physical exercise.[2] Exercise programs are suggested as helpful countermeasures against a large number of obesity-related disorders, including insulin resistance, dyslipidemia, diabetes, and NAFLD, that entail few side effects.[3]

In the context of the laboratory animal-based research, the Lieber-DeCarli diet is one of the high-fat-manipulated nutritional possibilities to induce obesity and related metabolic disorders in rodents, including NASH. However, despite rodents are frequently used as animal models to study the adverse effects of obesity, only limited information is yet available regarding the clinical and anatomical profile of rats fed with fat-rich diets. In addition, the time course of the distinct deleterious outcomes induced by this obesity-related nutritional model is not completely clear. Likewise, the impact of a preventive and a therapeutic exercise program against the obesity-related consequences of these diets has been scarcely studied.

Therefore, using the Lieber-DeCarli high-fat diet, we aimed to analyze the preventive and therapeutic effects of two exercise programs on the clinical and anatomical profile of obese rats.

2. Methods

2.1. Animals and diets

Male Sprague-Dawley rats with 5–6 weeks (125–150 g) purchased from Charles River (L'Arbresele, France) were individually housed in a normal environment (21–22 °C; 50–60% humidity, with 12 h light/dark cycles), receiving food and water *ad libitium*. Throughout the seventeen weeks of the experimental protocol, animals were fed with an isocaloric standard (containing 35% energy from fat, 47% from carbohydrates, and 18% from protein) or a high fat (71% energy derived from fat, 11% from carbohydrate, and 18% from protein) liquid diet purchased from Dyets Inc. (Bethlehem, USA) based on a previously described NASH model.[4] During the first week of the protocol, the standard diet was given to all animals as an adaptation to the liquid feeding. After this week, animals were randomly assigned into four groups: standard-diet sedentary (SS, $n = 20$), standard-diet voluntary physical activity (SVPA, $n = 10$), high-fat diet sedentary (HS, $n = 20$) and high-fat diet voluntary physical activity (HVPA, $n = 10$). After 9-weeks, half ($n = 10$) of the SS and HS animals were engaged in an ET program as follows: standard-diet endurance training (SET, $n = 10$) and high fat-diet endurance training (HET, $n = 10$).

The local Institutional Ethics Committee approved the present study, which followed the Guidelines for Care and Use of Laboratory Animals in research advised by the Federation of European Laboratory Animal Science Associations (FELASA). Several authors are accredited by FELASA to perform animal experimentation.

2.2. Exercise programs

The animals from voluntary physical active groups (SVPA and HVPA) were housed in cages equipped with running wheels and a digital counter, in which running distance was daily obtained between 08.00 and 10.00 h. The animals submitted to the endurance training protocol were adapted to the treadmill (SET and HET) for 5 days at 15 m/min and 0% grade until 30 min was achieved. The habituation week was followed by 8 weeks of endurance exercise during 5 days/week, 60 min/day at a starting speed of 15 m/min that was gradually increased until 25 m/min was reached. During the training protocol sedentary animals (SS and HS) were placed on a non-moving treadmill 5 days/week for 60 min in order to expose the animals to the same environmental and handling conditions.

2.3. Blood sampling and anatomical measurements

At 9 weeks of diet treatment, blood was collected from the tail of all animals, centrifuged (3000 rpm, for 10 min at 4 °C) and plasma was stored at −80 °C for later biochemical analysis. At the end of protocol (17 weeks) abdominal circumference (the largest zone of rate abdomen), body length (nose to anus) and weight were determined at the day of sacrifice with the animals anesthetized (Ketamine 90 mg/kg and Xylazine 10 mg/kg). Thereafter, blood was collected from the heart, centrifuged (3000 rpm, for 10 min at 4 °C) and the plasma was stored at −80 °C. The organs were then perfused with NaCl 0.9% and the liver, heart, femur, epididymal, retroperitoneal and mesenteric fat pads were excised and weighed. Epididymal fat was removed from areas surrounding the epididymis and testis. Retroperitoneal fat pad was taken as the deposit behind each kidney along the lumbar muscles. Mesenteric fat pad included the adipose tissue surrounding the gastrointestinal tract from the gastrooesophageal sphincter to the end of the rectum. Body mass index was calculated as the ratio between body length and weight (BMI = body weight (g)/body length^2 (cm^2)), while adiposity index was calculated as follows: (100·(sum of fat pad weights/body weight)). The food consumption was daily recorded and energy intake *per* week was calculated (1 ml = 1 kcal for both diets).

2.4. Light microscopy

Paraformaldehyde-fixed liver tissues were dehydrated in ethanol (70%–100%), and embedded in paraffin. The samples were then sectioned (5 μm), deparaddinized with xylene and stained with haematoxylin and eosin. After these proceedings, samples were blindly and randomly examined by light microscopy (Olympus BX61, Germany) by two pathologists in three sections *per* slide and classified according to the NAFLD activity score (NAS score). The NASH score was achieved by summing the scores of steatosis (0–3), lobular inflammation (0–2) and hepatocellular ballooning (0–2). A total score of 0–2 represents no steatohepatitis, 3-4 questionable and 5 or more, definite steatohepatitis.[5]

3. Results

3.1. Animal characteristics and treatment

As seen in Table 1, after 17 weeks of high-fat diet treatment, sedentary animals showed a significant increase in perirenal and mesenteric fat and in adiposity index (AI) (HS vs. SS). However, both exercise regimens diminished mesenteric fat and AI (VPA vs. HS and HET vs. HS), perirenal fat were only reduced in HET animals. Besides visceral adiposity, ET was also able to diminish body and liver weight, abdominal circumference and BMI, while increased heart-to-body weight ratios, in both diet types. These alterations were also observed in VPA animals in standard diet group. Surprisingly, despite a significant decrease of liver and body weight in HET animals, only a decreased tendency of liver/body weight ratio was found in HET compared to HS. No alterations were observed in energy intake, voluntary and treadmill running distances between groups (Fig. 1).

3.2. Morphological analysis

Liver micrographs from high-fat diet animals showed macro and microvesicular lipid droplets and hepatocyte ballooning (Fig. 2).

Table 1
Animal anatomic characteristics after 17 weeks of treatments.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[f]
Body weight (g)	676.8 ± 11.5	612.7 ± 15.3[a]	559.3 ± 10.0[a,c]	684.3 ± 14.2	710.0 ± 10.8[c]	592.8 ± 7.3[b,d,e]	DT × ET
Liver weight (g)	17.1 ± 0.3	16.2 ± 0.8	11.7 ± 0.7[a,c]	16.7 ± 0.5	16.8 ± 0.7	13.5 ± 0.5[b,d,e]	ET
Liver weight/Body weight (g g^{-1})	0.024 ± 0.001	0.027 ± 0.001	0.021 ± 0.001[c]	0.025 ± 0.001	0.024 ± 0.001	0.023 ± 0.001	ET
Heart weight/Body weight (g g^{-1})	0.0030 ± 0.0001	0.0032 ± 0.0002[a]	0.0036 ± 0.0001[a,c]	0.0026 ± 0.0001	0.0028 ± 0.0001[c]	0.0032 ± 0.001[b,d]	DT, ET
Femur length/Body weight (mm g^{-1})	0.63 ± 0.01	0.70 ± 0.01[a]	0.75 ± 0.01[a,c]	0.60 ± 0.02	0.61 ± 0.01[c]	0.72 ± 0.01[b,e]	DT × ET
Abdominal circumference (cm)	24.8 ± 0.3	23.3 ± 0.3[a]	21.9 ± 0.5[a]	25.8 ± 0.3	25.4 ± 0.4[c]	21.9 ± 0.5[b,e]	DT, ET
Body Mass Index (g/cm^2)	0.76 ± 0.02	0.74 ± 0.02	0.67 ± 0.01[a,c]	0.80 ± 0.02	0.81 ± 0.01[c]	0.71 ± 0.01[b,e]	DT, ET
Epididymal fat (g)	21.8 ± 1.4	17.4 ± 0.7	10.0 ± 0.6[a,c]	22.1 ± 3.4	18.3 ± 1.1	14.0 ± 0.6[b]	ET
Perirenal fat (g)	28.7 ± 0.9	20.6 ± 1.6[a]	12.8 ± 1.3[a,c]	36.1 ± 0.9[a]	33.2 ± 1.4[c]	20.7 ± 0.8[b,d,e]	DT, ET
Mesenteric fat (g)	10.4 ± 0.3	7.8 ± 0.8[a]	3.6 ± 0.6[a,c]	15.7 ± 0.8[a]	11.7 ± 0.8[b,c]	5.5 ± 0.2[b,d,e]	DT × ET
Adiposity Index %	9.0 ± 0.2	7.5 ± 0.5[a]	4.8 ± 0.5[a,c]	10.8 ± 0.4[a]	8.9 ± 0.4[b,c]	6.8 ± 0.1[b,d,e]	DT, ET

Values are means ± SEM, ($n = 10$). Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained ($p < 0.05$).
[a] vs. SS.
[b] vs. HS.
[c] vs. SVPA.
[d] vs. SET.
[e] vs. HVPA.
[f] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT × ET) or the absence of differences (NS) is shown.

Additionally, the applied NAFLD activity score system (NAS) demonstrated that both HS and HV groups developed a morphological phenotype consistent with NASH ET attenuated these histopathological features and the score classification of "definitive NASH" was downgraded to "questionable NASH".

3.3. Intermediate plasma analysis (at 9 weeks of combined treatments)

As shown from Table 2, after 9 weeks of high-fat diet a significant increase in plasma content of AST, ALT, cholesterol and HDL were observed in sedentary animal while TG and VLDL were

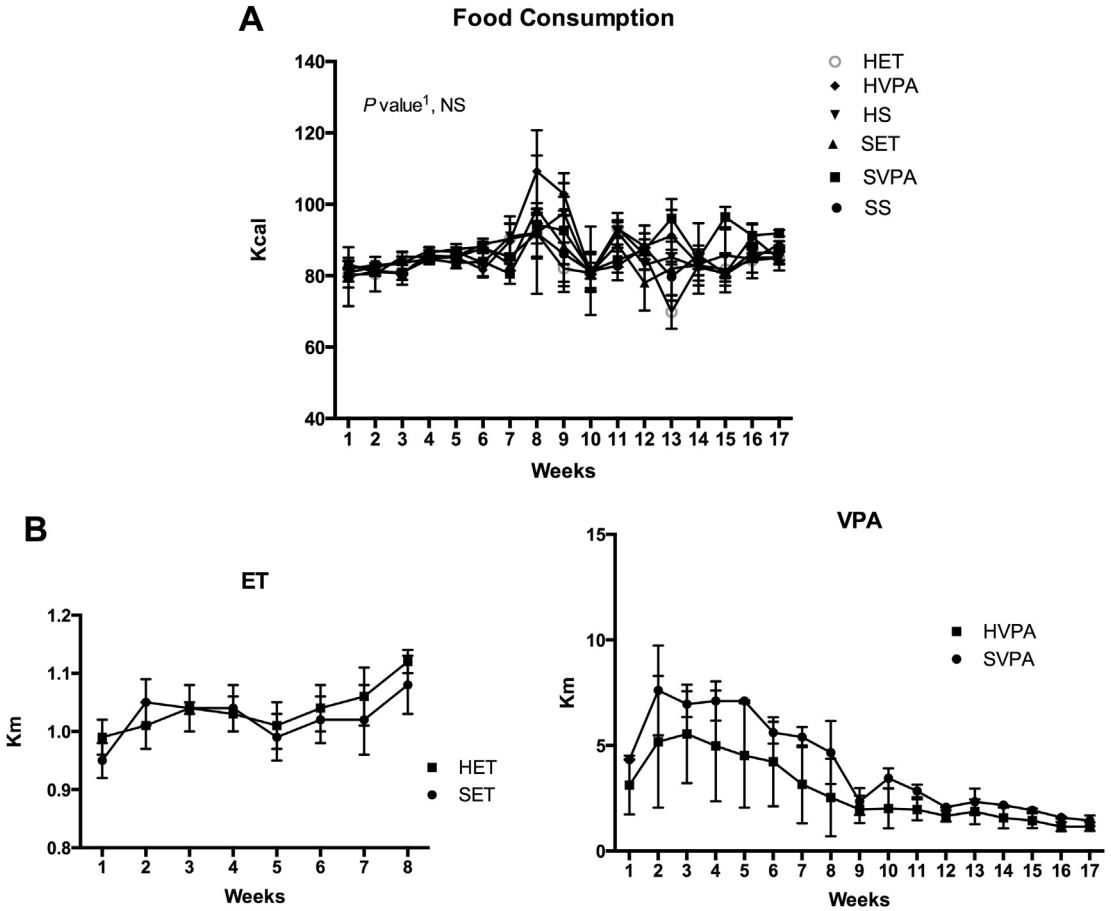

Fig. 1. Food consumption (A) and running distance in treadmill (ET) and free wheels (VPA) (B) *per week*. Values are Means ± SEM ($n = 10$). [1]Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT × ET) or the absence of differences (NS) is shown.

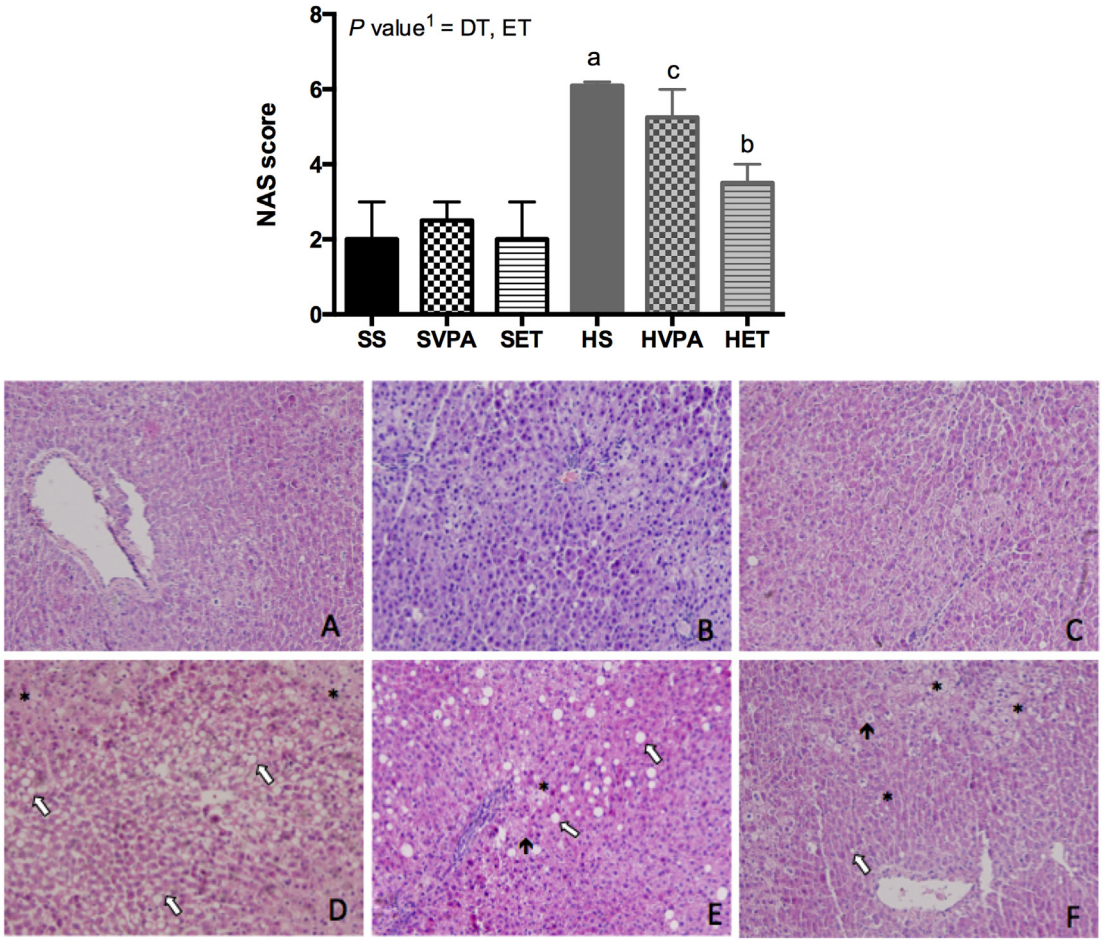

Fig. 2. Values are means SEM ($n = 10$) of NAS scores recorded from livers of experimental groups under light microscopy: (A), SS; (B) SVPA; (C) SET; (D) HS; (E) HVPA and (F) HET. NAS scores: 0-2 No NASH; 3-4 questionable NASH; >5 Definitive NASH (according to Kleiner et al.[5]). The presence of ballooning (asterisk), macro (white arrows) and microvesicular (black arrows) lipid droplets in high-fat diet groups. [a] vs. SS, [b] vs. HS, [c] vs. SVPA ($p < 0.05$). [1]Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT × ET) or the absence of differences (NS) are shown.

Table 2
Blood analysis at 9 weeks of treatments.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[f]
AST (U/L)	91.60 ± 1.60	96.75 ± 2.46	94.34 ± 2.22	105.0 ± 2.77[a]	90.75 ± 1.35[b]	102.90 ± 3.17[d,e]	DT × ET
ALT (U/L)	37.50 ± 1.78	36.25 ± 2.02	39.50 ± 0.39	50.83 ± 2.82[a]	40.80 ± 2.59[b]	51.43 ± 2.46[d,e]	DT, ET
Glucose (g/L)	113.00 ± 1.81	106.00 ± 4.45	111.20 ± 2.06	118.20 ± 2.65	125.25 ± 0.91[c]	119.10 ± 2.31[d]	DT × ET
Triglycerides (g/L)	41.40 ± 1.65	30.00 ± 1.47[a]	41.20 ± 0.91[c]	27.20 ± 1.14[a]	29.00 ± 1.27	26.43 ± 1.41[d]	DT × ET
Cholesterol (g/L)	60.50 ± 2.65	47.67 ± 2.39[a]	60.34 ± 3.11[c]	78.75 ± 2.41[a]	65.51 ± 2.07[b,c]	76.90 ± 2.21[d,e]	DT, ET
HDL (g/L)	36.60 ± 1.28	32.45 ± 0.49	37.00 ± 1.52	52.00 ± 1.29[a]	49.76 ± 2.73[c]	51.86 ± 1.10[d]	DT
VLDL (g/L)	8.28 ± 0.33	7.45 ± 0.32	9.76 ± 0.86[c]	5.44 ± 0.23[a]	8.35 ± 0.21[b]	6.14 ± 0.55[d,e]	DT × ET
C-reactive protein	0.23 ± 0.01	0.23 ± 0.01	0.20 ± 0.02	0.25 ± 0.01	0.22 ± 0.01	0.24 ± 0.01[d]	NS

Values are means ± SEM, ($n = 10$). Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained ($p < 0.05$).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; VLDL, very low-density protein.
[a] vs. SS.
[b] vs. HS.
[c] vs. SVPA.
[d] vs. SET.
[e] vs. HVPA.
[f] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT × ET) or the absence of differences is shown (NS) ≥ 0.05.

significantly decreased (HS vs. SS). VPA reverted most of the observed alterations (AST, ALT, VLDL) in these high-fat fed animals, although was only able to modulate TG and total cholesterol content in the animals treated with standard diet (SVPA vs. SS). Considering that ET animals only started the exercise protocol after this intermediate blood sampling, no alterations were observed between ET groups and sedentary controls (SS vs. ST and HS vs. HT).

3.4. Final plasma analysis (at 17 weeks of treatments)

After seventeen weeks of high-fat diet feeding, sedentary animals significantly increased HDL and diminished VLDL and TG plasma levels (HS vs. SS). Exercise regimens diminished TG (SVPA and SET) and cholesterol (SET) in standard diet feed animals, but no alterations were observed in HET and HVPA groups (Table 3).

Table 3
Blood analysis after 17 weeks of treatments.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[e]
AST (U/L)	131.70 ± 15.33	140.30 ± 27.91	131.0 ± 14.80	98.29 ± 10.95	131.50 ± 18.16	95.00 ± 2.96	DT
ALT (U/L)	38.25 ± 0.47	34.50 ± 3.43	34.40 ± 0.81	47.00 ± 6.98	45.00 ± 1.55	35.67 ± 2.28	NS
Glucose (g/L)	228.00 ± 18.68	217.00 ± 15.59	246.20 ± 12.79	248.30 ± 6.90	235.00 ± 12.51	212.10 ± 21.27	NS
Triglycerides (g/L)	106.50 ± 15.58	57.50 ± 5.55[a]	67.50 ± 9.30[a,b]	30.75 ± 2.55[a]	40.20 ± 6.99	22.86 ± 2.73[c]	DT × ET
Cholesterol (g/L)	69.00 ± 2.41	58.33 ± 2.46	51.40 ± 3.14[a]	77.51 ± 4.14	88.80 ± 8.51[b]	72.66 ± 3.93[c,d]	DT, ET
HDL (g/L)	35.00 ± 2.05	38.72 ± 2.05	34.36 ± 1.02	55.50 ± 3.00[a]	59.20 ± 4.76[b]	48.41 ± 2.51[c]	DT
VLDL (g/L)	17.28 ± 1.78	13.13 ± 0.51	19.07 ± 0.84[b]	6.15 ± 0.51[a]	8.04 ± 1.40[b]	4.57 ± 0.55[c]	DT
C-reactive protein	0.28 ± 0.02	0.31 ± 0.01	0.28 ± 0.02	0.29 ± 0.005	0.33 ± 0.01	0.29 ± 0.01	NS

Values are means ± SEM, ($n = 10$). Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained ($p < 0.05$).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; VLDL, very low-density protein.
[a] vs. SS.
[b] vs. SVPA.
[c] vs. SET.
[d] vs. HVPA.
[e] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT × ET) or the absence of differences (NS) is shown.

4. Discussion

Due to the alarming worldwide increase and its association with other chronic conditions such as diabetes, dyslipidemia, hypertension and NAFLD,[6] adulthood and children obesity is one of the most serious global health challenges of the 21st century. Therefore, the development of obese animal models has been a useful tool to understand the mechanisms involved in this pathology, as well as the effectiveness of some hypothetical strategies to prevent and mitigate the related side effects of obesity.

In 2004, Lieber-DeCarli[4] developed a nutritional *ad libitium* liquid diet able to induce obesity and NASH in animals. However, a deep understanding of the anatomical and clinical alterations induced by this high-fat diet model is still scarce. Moreover, although lifestyle modification focused on physical activity programs have been considered the first line therapy against obesity and metabolic-related diseases, both preventive (VPA) and the therapeutic (ET) effects of exercise have never been investigated so far in this particular diet model. Therefore, our study was designed to characterize the anatomical and clinical alterations induced by this Lieber-DeCarli diet model and to ascertain whether physical exercise used as preventive and therapeutic strategy could mitigate liver morphological abnormalities, altered blood lipid and transaminases profiles as well as some adiposity-related measures associated with this high-fat feeding model.

Our study suggests that Lieber-DeCarli diet induced increased visceral adiposity that was counteracted by both exercise interventions. The exercise model here used as a therapeutic approach (ET) positively modulated most of the anatomical/adiposity measures of obesity (body and liver weight, BMI, abdominal circumference) as well as the evident histological signs of NASH. In contrast, VPA did not prevent these features in the same extent.

4.1. Anatomical parameters

Several studies suggest that an increase of fat intake associated with sedentary behaviors is responsible for diet-induced overweight and obesity (for refs see 7). Moreover, it has been reported that body weight and distribution are dependent on the quality of diet fat (saturated, unsaturated, polyunsaturated fatty acids).[8] In the present study, no alterations between standard and high-fat diet treated animals were observed in caloric intake, body and liver weights, BMI and abdominal circumference. These results, which are in accordance with previous studies,[4,9] were somewhat expectable since diets are isocaloric. Despite the anatomical measurements of obesity remain unaltered; adipose tissue (mesenteric and perirenal) was significantly increased in HS animals (HS vs. SS), which could be related to the higher fat availability on this diet (71% vs. 35% of calories derived from fat). In fact, Estrandy et al.[10] observed that isocaloric diets rich in fat increased adiposity depot, without changes in body weight. Furthermore, the authors reveled that diet fatty acid content was reflected in fatty acid tissue composition. In parallel with overnutrition, physical inactivity has contributed to the spread of worldwide obesity and related comorbidities.[11] In the context of the animal-based studies, few have addressed the impact of exercise programs against high-fat diet induced obesity and NAFLD.[12–14] Our study showed that ET diminished most of the anatomical measures in high-fat diet groups, whereas VPA was only able to positively modulate tissue adiposity. This might be related to the higher exercise intensity achieved during the ET program compared to VPA. In fact, a growing volume of evidence reveal that exercise intensity has greater influence in the management of body weight and composition than self-regulated voluntary physical activity.[15]

4.2. Histological analysis

Since overweight is one of the main risk factors for NAFLD development, several obesity-related nutritional and genetic models have been used to induce hepatic damage in rodent. The main disadvantage of genetic models such as ob/ob and Db/db rats is that they also need to be fed with a high-fat or methionine and choline deficient diet to develop NASH,[16] making these models even more expensive. Regarding the nutritional models, several promising results were obtained with intragastric overfeeding of animals, since they mimic the histopathological and pathogenic features of NASH.[17] However, these models require technical expertise, specialized equipment and forced overfeeding, which can contribute to additional stress in animals.[9] Considering this, Lieber et al.[4] proposed a high-fat diet model that reproduces the most histopathological and major biochemical features observed in NASH patients through *ad libitium* consumption. As expected, our results are in agreement with Lieber group[4] findings, showing that high-fat fed in sedentary animals induced the typical hepatic lesions of NASH, namely steatosis, hepatocellular ballooning and clear signs of inflammation. Our study showed that ET, but not VPA, was able to positively counteract the hepatic “architecture” alterations induced

by the high-fat diet regimen. In fact, the histological analysis revealed that ET animals were absent of collagen deposition (data not shown), had a lower number of lipid droplets and ballooning hepatocytes. On the other hand, VPA animals maintained evidences of liver degeneration, such as macrovesicular steatosis, ballooning and the presence of collagen fibers (data not shown). The distinct effect of the preventive (VPA) and therapeutic (ET) approaches on these parameters may be related, at least in part, to the major anatomical alterations observed in ET animals compared to VPA counterparts. In fact, previous reports in NAFLD/NASH patients demonstrated that exercise-based lifestyle intervention, in which weight or abdominal circumference decreased, concomitantly improve liver morphology.[18,19] However, unexpectedly, despite the significant decrease in liver and body weight in HET compared to HS, only a decreased tendency was found in the liver/body weight ratio. It is our belief that besides systemic alterations, some tissue-related mechanisms involved in liver fat removal and oxidation, such as mitochondrial function[20] and lipophagy[21–23] might have contributed to improve liver morphology. Moreover, in accordance with findings regarding the effect of the two distinct isocaloric diets used in this study, e.g., no significant differences in liver, body weight and in liver/body weight, but consistent and significant differences in histological features between the diet groups as previously reported by others,[4,9,24,25] exercise might also had positively modulated liver anatomical and physiological characteristics without obvious repercussions in liver weight. For these reasons, this ratio should probably be used with caution as a marker of liver (re)modeling under physiological or pathological conditions.

4.3. Serum markers

The diagnosis of NAFLD/NASH is usually suspected in the presence of other pathologies, such as obesity, diabetes and obstructive sleep apnea; however since the majority of patients are asymptomatic, abnormal liver function is only incidentally found during routine blood analysis.[26] Among several available serum markers, alterations in aminotransferase activity are traditionally accepted as a clue for hepatic injury.[27] The upper normal limit for serum ALT concentrations has been set around 30–40 IU/L.[28] In our study, after 9 weeks of high-fat diet, ALT and AST contents were significantly increased in sedentary animals (HS vs. SS). However, these alterations were not present at 17 weeks of diet treatment, which is not uncommon in NAFLD patients. In fact, several studies proposed that the histological spectrum of NAFLD could be observed in patients with normal ALT values.[29] These contradictory results between the 9th and 17th weeks underlined the hypothesis that ALT and AST are non-reliable markers of NASH or fibrosis.[30] Accordingly with previous data,[31] the diminishing of circulating VLDL levels observed at 9th and 17th weeks reinforce the possibility of hepatic injury as blockage of VLDL secretion seems to contribute to fat storage in the liver and thus to NAFLD development. Moreover, the total plasma cholesterol levels, described as strong risk factor for the development of NAFLD/NASH,[32] were also significantly increased in HS after 9th weeks of high-fat diet treatment. The unexpected increase of HDL could be related to the used polyunsaturated fatty acids-enriched diets,[33] while the reduced TG content may be a consequence of the possible impairment in VLDL export and/or increased clearance of TG by peripheral tissues.[34] Despite exercise has been described as an effective strategy against several deleterious effects associated with NAFLD and related metabolic disorders,[18,19,35] VPA was only able to positively modulate aminotransferases (ALT and AST), total cholesterol and lipoproteins (VLDL) levels in blood at 9th week. Unfortunately, the inability of exercise-based lifestyle strategies to ameliorate the lipid profile is not uncommon. A recent study suggested that 16 weeks of endurance training performed by obese NAFLD patients, promoted a beneficial effect in intrahepatic triglyceride content, although without changes in lipoprotein kinetics.[36]

5. Conclusion

In conclusion, data confirmed that exercise is a promising strategy for counteracting the anatomical and histological features of Lieber-DeCarli diet-induced obesity and related metabolic disorders including NASH. ET used in a therapeutic context attenuated the disease phenotype, while VPA used as a preventive tool only counteracted some features of the disease progression. Considering that VPA was performed during 17 weeks and ET only during 8 weeks, and the accumulated distance covered by VPA is much higher than ET, this major protection is probably related, at least in part, to the higher exercise intensity achieved in forced exercise compared with self-regulated VPA. Therefore, the present study reinforces that exercise intensity, more than total volume, should be considered in exercise counseling and intervention programs for NASH treatment.

Statement of authorship

All the authors were significant contributors, providing the concept, as well as the experimental design of the study and the writing of the manuscript. Moreover, all authors performed the functional and biochemical experiments and contributed to the interpretation of the results and the critical revision of the manuscript draft.

Sources of founding

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Conflict of interest

None.

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Role of physical exercise on hepatic insulin, glucocorticoid and inflammatory signaling pathways in an animal model of non-alcoholic steatohepatitis

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ABSTRACT

Aims: Pro-inflammatory mediators, glucocorticoids and transforming growth factor (TGF)-β are implicated in the pathogenesis of non-alcoholic steatohepatitis (NASH)-related insulin resistance. As physical activity is beneficial against NASH, we analyzed the voluntary physical activity (VPA) and endurance training (ET) (preventive and therapeutic strategies) effects on hepatic insulin, pro-inflammatory and glucocorticoid signaling regulators/mediators in high-fat (Lieber-DeCarli) diet (HFD)-induced NASH.
Main methods: Adult male Sprague–Dawley rats were divided in standard diet (SD) or HFD, with sedentary, VPA and ET animals in both diet regimens. Plasma glucose and insulin concentrations were analyzed; plasma insulin sensitivity index (ISI) was calculated. Hepatic insulin, pro-inflammatory and glucocorticoid signaling regulators/mediators were evaluated by Western blot or reverse transcriptase-PCR.
Key findings: ET improved ISI in both diet regimens. HFD-feeding increased interleukin-1β and induced a similar pattern on interleukin-6 and TGF-β, which were globally reduced by physical exercise. ET decreased HFD leukemia inhibitory factor level, SD + VPA animals presenting higher values than HFD + VPA animals. HFD increased the ratio of IRS-1^{Ser307}/total IRS-1, which was completely mitigated by physical exercise. Physical exercise reduced total ERK and JNK (total and activated) expression in HFD. In SD *vs.* HFD, VPA presented higher activated JNK and ET presented higher total JNK. Generally, in HFD, the ratio (activated/total) of AKT, and each separately, decreased with exercise and also for activated AKT in SD. Overall, in both diets, exercise reduced 11β-hydroxysteroid dehydrogenase type 1. ET increased glucocorticoid receptor and reduced PTP1B in HFD.
Significance: Physical exercise mitigates the expression of pro-inflammatory mediators and positively modulates insulin and glucocorticoid signaling in NASH.

Introduction

The prevalence of NASH, a progressive form of non-alcoholic fatty liver disease (NAFLD), increased dramatically in the last few years as a consequence of excessive consumption of high-caloric food and/or sedentary life style [1,2]. NASH is characterized, among other factors, by aberrant hepatic lipid droplet accumulation, pro-inflammatory cellular environment and insulin resistance [1–4].

Although the precise mechanisms underlying NASH-related hepatic insulin resistance are not yet completely understood, several studies implicate hepatic inflammation and ectopic deposition of fat as well as hepatic increased levels of glucocorticoids and increased TGF-β signaling in the process [3–8].

Hepatic inflammation, mediated by nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), NF-κB inhibitor (IκB) kinase (IKK) and/or c-Jun N-terminal kinase (JNK) pathways, coupled with hepatic ectopic lipid deposition (coursing with diacylglycerol and ceramide accumulation), induces and/or exacerbates hepatic insulin resistance [5,6,9–12]. Additionally, increased protein tyrosine phosphatase 1B (PTP1B, the major regulator of the insulin signaling pathway) expression has been observed in liver biopsies of NASH patients [13] as well as in the liver of HFD-fed mice [14], which can be linked to hepatic insulin resistance [14–18] and inflammation, since tumor necrosis

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factor alpha (TNF-α) induces both mRNA and protein expression of hepatic PTP1B via nuclear NF-κB activation [14]. Both PTP1B and 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) are abundant enzymes located/tethered on the cytosolic surface of the endoplasmic reticulum (ER) [17].

11β-HSD1 regulates tissue glucocorticoid action, at the pre-receptor level, through the conversion of inert cortisone into biologically active cortisol in humans (or dehydrocorticosterone into corticosterone in rodents). Glucocorticoid receptor (GR) and sirtuin 1 (Sirt1) are important in glucocorticoid signaling [3,7,19–21]. Increased glucocorticoid signaling is associated with insulin resistance [7,20,22]. Additionally, hepatic mRNA expression and/or activity of 11β-HSD1 and GR parallels NASH progression, which implies a role in response to chronic inflammation and suggests that glucocorticoid metabolism is critical in the onset and/or progression of NASH [3]. Likewise, increased hepatic TGF-β signaling is closely associated with hepatic insulin resistance and contributes to hepatic steatosis, inflammation, fibrosis and hepatocyte death [4,8,23,24], suggesting TGF-β as a relevant contributor to the progression of NASH [25].

Several epidemiological and observational studies suggest that physical inactivity is associated with obesity, systemic inflammation, hepatic insulin resistance and type 2 diabetes mellitus [26,27]. On the other hand, regular exercise training is widely accepted as a non-pharmacological tool against isolated features included in NASH, such as hepatic steatosis and hepatic inflammation, in both humans and rodents. Additionally, regular exercise training improves insulin sensitivity in the liver and glucose homeostasis [26,28,29]. However, the impact of physical exercise on the specific context of NASH-related hepatic insulin, glucocorticoid and pro-inflammatory signaling pathways is undetermined so far. Therefore, the objective of this work was to analyze the effects of VPA and ET (preventive and therapeutic strategies) on hepatic insulin, pro-inflammatory and glucocorticoid signaling regulators/mediators in HFD (Lieber-DeCarli)-induced NASH. Here, in an animal model of HFD-induced NASH, we found that both VPA and ET had positive effects on those signaling pathways.

Materials and methods

Treatment of animals

Thirty-six male Sprague–Dawley rats (aged 5–6 weeks and weighing 125–150 g) were purchased from Charles River (L'Arbresle, France). Animals were individually housed in cages (with an enriched environment) and maintained in a temperature and humidity-controlled room (21–22 °C; 50–60% humidity) with reversed 12 h light/dark cycles, with *ad libitum* access to water and food (provided in the liquid state).

As shown in Fig. 1, the feeding and activity protocols were preceded by a 1-week period for adaptation to the liquid diet, in which standard control liquid diet was given to all animals. Then, the animals were randomly ascribed into six groups as follows: standard liquid diet + sedentary animals (SS), standard liquid diet + voluntarily physically active animals (SVPA), standard liquid diet + endurance trained animals (SET), liquid HFD + sedentary animals (HS), liquid HFD + voluntarily physically active animals (HVPA) and liquid HFD + endurance trained animals (HET) [30]. The liquid HFD [previously described to induce NASH [31] provided 71% of energy from fat, 11% from carbohydrate and 18% from protein (Lieber-DeCarli diet #712031) and the isocaloric standard control liquid diet provided 35% of energy from fat, 47% from carbohydrate and 18% from protein (Lieber-DeCarli diet #710027); both diets were purchased from Dyets Inc. (Bethlehem, PA, USA). The study was approved by local Institutional Ethics Committee and followed the guidelines for the care and use of laboratory animals in research advised by the Federation of European Laboratory Animal Science Associations (FELASA) and Portuguese Act 129/92. Several authors of this manuscript are accredited by FELASA to perform animal experimentation.

Animals of the SVPA and HVPA groups had free access to a free wheel throughout the 17 weeks of the protocol and the running distance was obtained daily from a digital counter between 08.00 and 10.00 am. After 8 weeks of diet consumption, half of the SS and HS animals were submitted to endurance training (SET and HET, respectively) while the other half continued to be sedentary. Initially, SET and HET rats were progressively acclimated to the motor driven treadmill for 5 days/week at 15 m/min and 0% grade until 30 min of running was achieved. One-week acclimation was followed by 8 weeks of endurance exercise for 5 days/week, 60 min/day at a starting speed of 15 m/min, which was gradually increased over the training program until 25 m/min was reached. Sedentary animals were placed on a non-moving treadmill 5 days/week for 60 min in order to expose the sedentary animals (SS and HS) to the same environmental conditions but without promoting any physical training adaptation.

Sacrifice, blood samples and liver extraction of animals

Rats were deeply anesthetized with a ketamine/xylazine combination [90 mg/kg of ketamine (Merial, Lyon, France) and 10 mg/kg of xylazine (Bayer, Lisbon, Portugal)]. Blood was collected from the left ventricle and plasma was separated (5000 *g* for 5 min at 4 °C), split into aliquots and stored at −80 °C for later biochemical analyses.

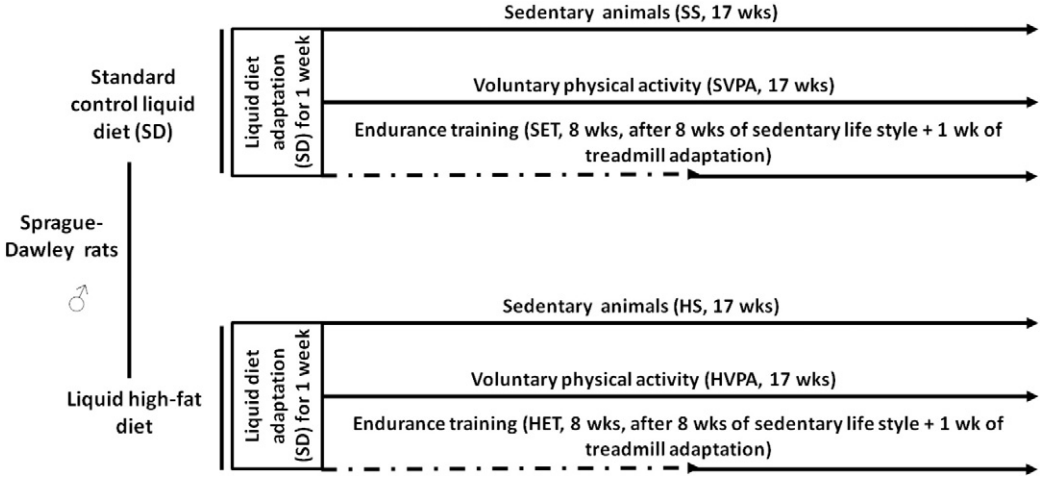


Fig. 1. Design of the diet and exercise training protocol. HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; SD, standard control liquid diet; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet; wk(s), week(s).

Animals were transcardiacally perfused with an ice-cold isotonic sodium chloride solution. After perfusion, the liver was rapidly removed from the abdominal cavity, washed in the same solution and cut in several fragments. The liver fragments were stored at −80 °C, until polymerase chain reaction (PCR) and immune-blotting (Western blot; WB) analyses were performed.

Evaluation of plasma biochemical parameters

Plasma glucose concentration was quantified in the Clinical Pathology Unit of São João Hospital Centre, EPE, Porto, Portugal, using a standardized method for an automated clinical chemistry analyzer (Olympus AU54001, Beckman-Coulter1, Izasa, Porto, Portugal). Insulin quantification was performed using the ultrasensitive Mercodia ELISA Kit (10-1137-01 Kit, Uppsala, Sweden), according to the manufacturer's instructions. ISI calculation was adapted from Cacho and co-workers [32].

RNA extraction and semi-quantitative reverse transcriptase-PCR

Total RNA was extracted from liver samples using a Tripure Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), dissolved in diethylpyrocarbonate-treated water and stored at −80 °C, until further analysis. Quantity and quality of isolated RNA were assessed using a NanoDrop™ 1000 spectrophotometer by reading absorbance at 230 nm, 260 nm and 280 nm (Thermo Scientific, CA, USA); samples with ratios of $A_{260}/A_{280} > 1.9$ were used for cDNA synthesis. Two point five micrograms of total RNA were used as template for cDNA production using the NZY First-strand cDNA Synthesis Kit (NZYTech, Lda, Lisbon, Portugal; https://www.nzytech.com/site/cDNA-Synthesis-kits/NZY-First-Strand-cDNA-Synthesis-Kit), which included a combination of random hexamers and oligo$(dT)_{18}$ primers. The primers were included in the NZYRT 2× Master Mix, which also contained dNTPs, $MgCl_2$ and an optimized reverse transcriptase (RT) buffer. The NZYRT Enzyme Mix included both NZY RT and NZY ribonuclease inhibitor. RNA samples were incubated with the NZYRT Enzyme Mix, supplemented with the NZYRT 2× Master Mix, for 10 min at 25 °C, 30 min at 50 °C, followed by heat-inactivation of the reaction, at 85 °C for 5 min, and then chilled on ice. After that, samples were incubated for 20 min at 37 °C with 1 μL RNAse H (*Escherichia coli*). The RT product was stored at −20 °C until required. Using 2 μL of RT product, PCR amplification was performed in the presence of 2.3 mmol/L of $MgCl_2$, 0.5 mmol/L of each primer, 0.2 mmol/L dNTPs, 2 U of supreme Taq DNA polymerase in a final volume of 25 μL. Simultaneous amplification of the invariant housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was performed. Amplification started with a denaturation step at 94 °C for 5 min, preceding the amplification cycles after which there was a final elongation period at 72 °C for 10 min. Primer sequences, expected PCR product sizes and PCR conditions are depicted in Table 1.

Individual PCR reaction products were run on 2% agarose gel electrophoresis supplemented with ethidium bromide and visualized with an ultraviolet transilluminator (VilberLourmat, Marne La Vallée, France). The transcription expression of each of the genes evaluated was quantified and normalized to GAPDH expression of the same sample using VisionWorks®LS Software (VWR International, Leuven, Belgium).

Western blot analysis

Portions of liver were processed for total protein extraction through mechanical homogenization in a cold lysis buffer solution containing 50 mM Tris (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1% deoxycholic acid sodium salt, in the presence of phosphatase inhibitors [phosphatase inhibitor cocktails 1 and 2, P5726 and P0044, respectively, from Sigma-Aldrich (Barcelona, Spain)] and protease inhibitors (protease inhibitor cocktail, P8340, also from Sigma-Aldrich). After centrifugation at 14,000 *g*, for 10 min, supernatants were collected and protein content was quantified using the Bradford method [33]. Fifty micrograms of each sample were separated by electrophoresis in SDS-polyacrylamide gels (ranging from 6% to 12%), as described by Laemmli [34], followed by blotting onto a nitrocellulose membrane (Hybond; Amersham GE Healthcare, Buckinghamshire, UK) according to the method of Locke and co-workers [35]. Then, the membranes were stained with Ponceau S to evaluate the quality of protein transfer. Membranes were washed with TBST (Tris-buffered saline with Tween 20) to remove Ponceau S and non-specific binding was blocked with 5% non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or 5% bovine serum albumin (BSA; NZYTech, Lda) in TBST for 1 h, at room temperature. After that, the membranes were incubated overnight at 4 °C with specific primary antibodies diluted in 2.5–5% non-fat dry milk or 2.5–5% BSA in TBST: insulin receptor substrate-1 (IRS-1; 1:200 dilution; sc-8038, Santa Cruz Biotechnology, CA, USA), p-IRS-1 (1:200 dilution; Ser307; sc-33956, Santa Cruz Biotechnology, CA, USA), insulin receptor substrate-2 (IRS-2; 1:200 dilution; sc-1555, Santa Cruz Biotechnology, CA, USA), JNK (1:500 dilution; sc-7345, Santa Cruz Biotechnology, CA, USA), p-JNK (1:100 dilution; Thr183 and Tyr185; sc-6254, Santa Cruz Biotechnology, CA, USA), protein kinase B (serine/threonine-specific protein kinase; AKT1/2/3;1:3500 dilution; sc-8312, Santa Cruz Biotechnology, CA, USA), p-AKT1/2/3 (1:200 dilution; Ser473; sc-33437; Santa Cruz Biotechnology, CA, USA), extracellular-signal related kinase (ERK1/2; 1:200 dilution; sc-135900, Santa Cruz Biotechnology, CA, USA), p-ERK1/2 (1:200 dilution; Thr202 and Tyr204; sc-81492, Santa Cruz Biotechnology, CA, USA), IKK (1:1000 dilution; E021123; EnoGene Biotech Co. Ltd, NY, USA), NF-κB (1:2000 dilution; p65-Rel A; p/n 100–4165; Rockland Immunochemicals Inc., PA, USA), interleukin-1β (IL-1β; 1:200 dilution; sc-7884, Santa Cruz Biotechnology, CA, USA), interleukin-6 (IL-6 1:500 dilution; Ab-6672, Abcam Biotechnology, CA, USA), TGF-β (1:450 dilution; Ab-66043, Abcam Biotechnology, CA, USA), leukemia inhibitory factor (LIF; 1:200 dilution; sc-20087; Santa Cruz Biotechnology, CA, USA), 11β-HSD1 (1:1000 dilution; sc-20175; Santa Cruz Biotechnology, CA, USA), GR (1:500 dilution; sc-1004; Santa Cruz Biotechnology, CA, USA) and Sirt1 (1:400; sc-15404, Santa Cruz Biotechnology, CA, USA).

Antibody binding was detected after incubation with the respective secondary anti-rabbit (sc-2317; Santa Cruz Biotechnology, CA, USA), anti-mouse (sc-2314; Santa Cruz Biotechnology, CA, USA) or anti-goat (sc-2020; Santa Cruz Biotechnology, CA, USA) IgG antibodies for 1 h at room temperature and using enhanced chemiluminescence (ECL kit; Amersham Biosciences, Buckinghamshire, UK).

Membranes were stripped and re-probed with a goat anti-rat β-actin antibody (1:2000 dilution; sc-1616; Santa Cruz Biotechnology, CA, USA) and respective secondary anti-goat (sc-2020; Santa Cruz Biotechnology, CA, USA), as described above.

Table 1
Primer sequences, expected PCR product sizes and PCR conditions.

Genes	Primer sequence (5′-3′)	Denaturation	Annealing	Elongation	Cycles
PTP1B (408 bp)	F - GACCCGTCCTCTGTGGACATCAA R - ACCCACCATCCGTTTCCTAACT	94 °C, 45 s	58 °C, 45 s	72 °C, 45 s	30
GAPDH (683 bp)	F - ACTGGCGTCTTCACCACCAT R - TCCACCACCCTGTTGCTGTA	94 °C, 45 s	58 °C, 45 s	72 °C, 45 s	30

bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTP1B, protein tyrosine phosphatase 1B.

Alterations in proteins levels were detected by ChemiDoc™ XRS + System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to the level of β-actin of the same sample using Image Lab™ software 4.0.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Values are presented as mean ± standard error of the mean (SEM). The statistical significance of differences among groups was evaluated using the Kruskal–Wallis test followed by Dunn's Multiple Comparison Test. Statistical analysis was performed using GraphPad Prism® software (GraphPad Version 6.0, La Jolla, CA, USA). Differences between experimental groups were considered significant with a confidence interval of 95%, whenever $p < 0.05$.

Results

Plasma glucose homeostasis

As can be depicted in Fig. 2, no significant differences were found between groups for plasma glucose (Fig. 2A) and insulin (Fig. 2B) levels. However, ET seemed to decrease plasma insulin levels, in a similar manner, in animals fed with standard diet or HFD when compared with their sedentary and VPA counterparts, which most probably strongly contributed to the ISI results. ISI (Fig. 2C) was higher in ET animals compared with sedentary and VPA groups in both diet regimens (significantly for SET compared with SS as well as for HET compared with HVPA).

Hepatic pro-inflammatory parameters

No significant differences were observed in the protein content of IKK (Fig. 3A) and NF-κB (Fig. 3B).

IL-1β (Fig. 3C), IL-6 (Fig. 3D) and TGF-β (Fig. 3F) protein content presented similar variation patterns: remaining almost unchanged among groups fed with the standard diet and increasing in HS *vs.* SS (significantly for IL-1β), which was mitigated by both ET and VPA (with just a tendency being observed for HVPA *vs.* HS for IL-6).

Likewise, both VPA and ET decreased LIF protein content (Fig. 3E) in HFD-fed animals (significantly in HET *vs.* HS). HFD *vs.* standard diet did not increase LIF protein content in the sedentary animals. For both LIF and TGF-β, the HVPA and HET groups showed lower values when compared with SVPA and SET, respectively (significantly only for the former parameter in the VPA groups).

Hepatic insulin signaling mediators

Although no significant differences among groups were found in the protein content of inhibitory phosphorylated IRS-1 (Fig. 4A) or total IRS-1 (Fig. 4B), the ratio of inhibitory phosphorylated IRS-1 to total IRS-1 was significantly higher in HS when compared with SS (Fig. 4C). In addition, data clearly showed that both VPA and ET interventions were efficient in reverting the ratio of inhibitory phosphorylated IRS-1 to total IRS-1 back to levels similar to those found in standard diet animals (Fig. 4C).

Protein content of IRS-2 was similar in all groups independently of the diet regimen or the physical exercise strategy (Table 2).

ET significantly decreased PTP1B mRNA expression when compared with sedentary and VPA counterparts in HFD regimen (Table 2).

HFD did not induce any alteration in the levels of activated JNK or total JNK in sedentary animals (HS *vs.* SS) (Fig. 4D and E, respectively). VPA and ET significantly decreased activated JNK and total JNK levels in HFD (Fig. 4D and E, respectively). Exercised HFD-fed animals presented lower values than exercised standard diet-fed groups (significantly for HVPA *vs.* SVPA and HET *vs.* SET for activated JNK (Fig. 4D) and total JNK (Fig. 4E), respectively). Both exercise programs induced a decreasing pattern for total JNK in standard diet-fed animals (Fig. 4E). No significant differences were found among groups in the ratio of activated JNK to total JNK (Fig. 4F).

Diet had no effect on activated AKT (Fig. 4G) or total AKT (Fig. 4H) protein content as well as on p-AKT/AKT (Fig. 4I). In both diet regimens, we observed an identical variation pattern for activated AKT: when considering sedentary animals, both VPA and ET routines clearly reduced its expression (significantly for ET) (Fig. 4G). VPA and ET significantly reduced total AKT in HDF-treated rats (with a similar pattern in the standard diet) (Fig. 4H). The ratio of activated AKT to total AKT (Fig. 4I) was lower in exercised HFD-fed animals compared with the sedentary group (significantly for HET *vs.* HS).

No alterations by HFD in activated ERK (Fig. 4J) and total ERK (Fig. 4K) protein content were observed (HS *vs.* SS). Nevertheless, overall, both VPA and ET interventions seemed to decrease the expression of total ERK (Fig. 4K) and activated ERK (Fig. 4J), significantly for total ERK in HFD-fed animals. No significant differences were found among groups regarding the ratio of p-ERK/ERK (Fig. 4L).

Hepatic glucocorticoid signaling mediators

The 11β-HDS1 protein content (Fig. 5A) was not altered by the diet regimen *per se*. On the other hand, VPA and ET significantly reduced 11β-HDS1 protein content in liver of rats treated with both diets (the exception being a similar variation pattern for HET *vs.* HS). Likewise,

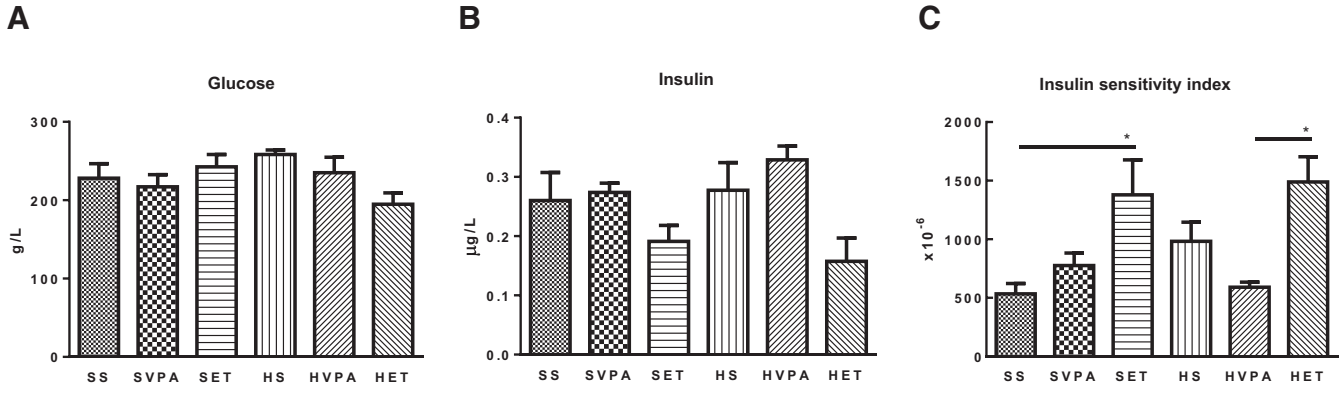


Fig. 2. The effect of diet, voluntary physical activity and endurance training on plasma glucose homeostasis: A) glucose (g/L); B) insulin (μg/L); C) insulin sensitivity index. Values are expressed as mean ± SEM. HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet. *$p < 0.05$.

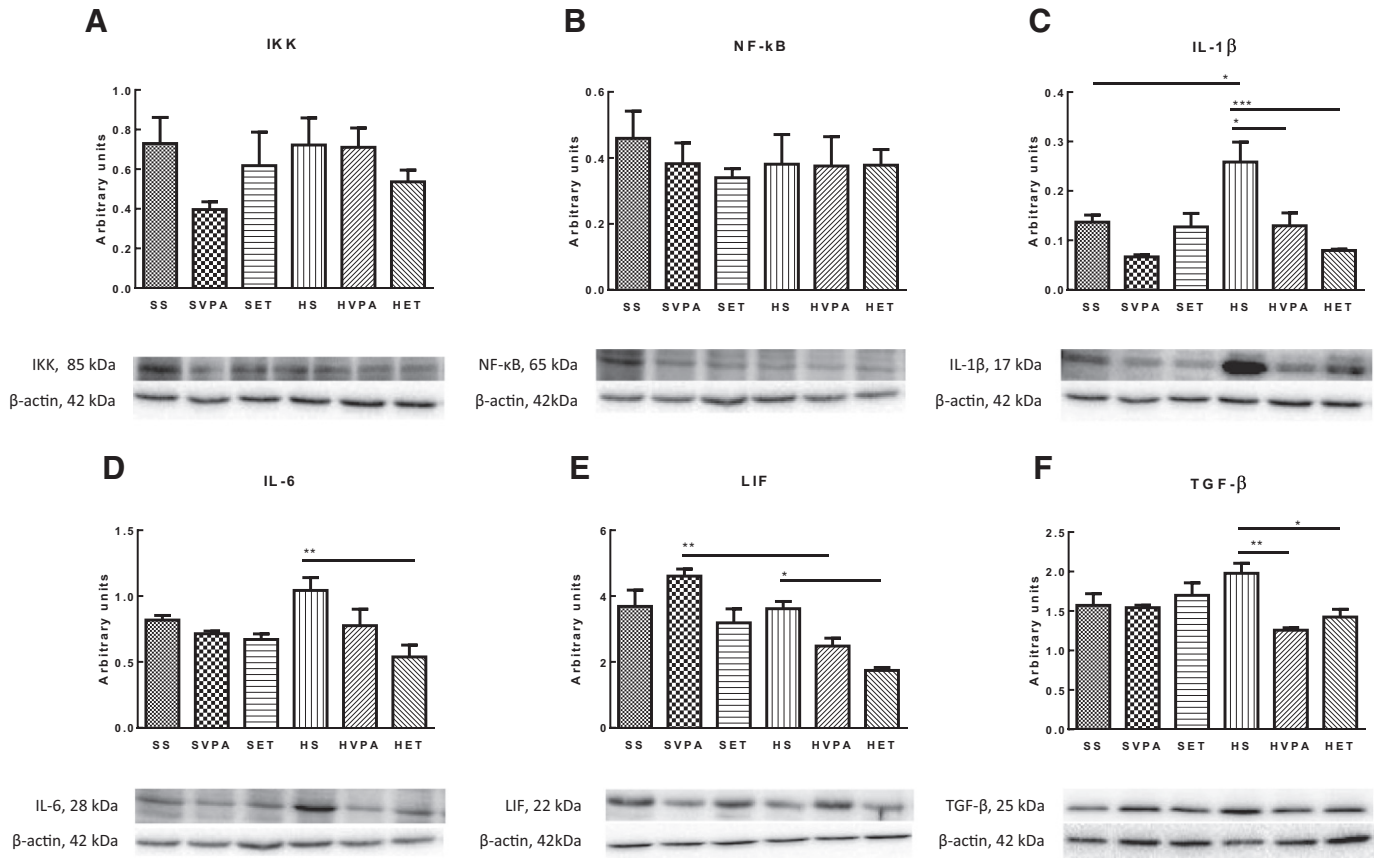


Fig. 3. Effects of diet, voluntary physical activity and endurance training on hepatic inflammatory signaling parameters. Representative bands obtained by semiquantitative Western blot analysis of A) NF-κB inhibitor (IκB) kinase (IKK), B) nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), C) interleukin-1β (IL-1β), D) interleukin-6 (IL-6), E) leukemia inhibitory factor (LIF), and F) transforming growth factor-β (TGF-β). Values are expressed as mean ± SEM. HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; kDa, kiloDalton; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

the HFD *per se* did not alter the GR protein content (Fig. 5B), but ET clearly increased GR levels in HFD (a parallel variation pattern was observed in the standard diet). No significant differences among groups were observed for Sirt1 protein expression (Fig. 5C).

Discussion

Regular physical exercise has a strong and positive impact against NASH development and progression [29,30,36,37]. However, the molecular mechanisms associated with these beneficial effects are not yet completely elucidated. As insulin and glucocorticoid signaling and pro-inflammatory pathways have been implicated in the pathogenic effects of HFD-induced NASH, protein content or mRNA expression of several of their regulators/mediators was evaluated in animals submitted to ET or VPA, therapeutic or preventive strategies, respectively.

In accordance with the histological score from Kleiner and coworkers [38], we have recently reported that 17 weeks of HFD resulted in NASH [30]. Generically, HFD-induced NASH results from an imbalance between energy intake and expenditure, with physical exercise often being considered an efficient intervention to mitigate this pathological condition [29,30,37]. We have shown that ET is able to attenuate NASH-morphological alterations (as confirmed by light electron microscopy) [30]. Moreover, the distinct effects of ET and VPA in the histological liver phenotype [30] may have resulted from the distinct exercise intensities. In this regard, high-intensity exercise, but not high-volume, has been inversely correlated with NAFLD severity [37].

In the present study, although VPA and ET did not decrease plasma glucose concentration in both diets, we observed a tendency for ET to reduce the plasma insulin levels that may have accounted for the significant ISI improvement observed in ET animals fed with both types of diets, as previously reported [28].

In our study, HFD significantly increased the ratio of IRS-1^{Ser307} to total IRS-1, as expected [39], suggesting an impairment of the insulin signaling pathway [40,41]. Interestingly, both VPA and ET completely prevented this HFD-related effect. In the HFD branch, both VPA and ET significantly reduced total JNK expression and JNK activation, thus suggesting that VPA and ET might have functioned as analogs of JNK inhibitors contributing to the reduction of the IRS-1^{Ser307} to total IRS-1 ratio induced by both VPA and ET in the HFD condition. In fact, an increased activation of the JNK pathway has been demonstrated to be directly implicated in the mechanism of HFD-induced hepatic insulin resistance through inhibitory serine phosphorylation of IRS-1, inhibiting the insulin-stimulated tyrosine phosphorylation of IRS-1 [5,40,42–45]. Additionally, in the HFD branch, both types of exercise decreased the p-AKT/AKT ratio, although reaching significance only for HET (*vs.* HS). In fact, increased basal and/or insulin-stimulated AKT activation, particularly in the liver, has been previously reported in different insulin resistant animal models and has been associated with an increase of oxidative stress, mitochondrial dysfunction and a worse metabolic profile [46–48]. Taking this into consideration, the greater decrease of AKT activation in HET (*vs.* HVPA) may have also contributed to the increase of ISI observed in this group.

Here, we showed for the first time, that both preventive and therapeutic interventions using VPA and ET, respectively, reduced the hepatic expression of 11β-HDS1 in an animal model of HFD-induced NASH. This finding is in complete accordance not only with the reduction in the ratio of inhibitory phosphorylated IRS-1 to total IRS-1 but also with the reduction of pro-inflammatory mediators induced by physical exercise, which reinforces the idea that hepatic insulin signaling, inflammation and 11β-HDS1 protein content are tightly interconnected [49–52] and involved in the positive role of physical exercise on the liver from HFD-induced NASH. Glucocorticoids increase serine^{307} phosphorylation of IRS-1 and, consequently, decrease the affinity of IRS-1 for the insulin receptor and increase IRS-1 degradation in skeletal muscle [53,54] and, conversely, 11β-HDS1 deletion and/or inhibition is associated with improved insulin sensitivity [49,52,53,54,55]; in skeletal muscle, selective 11β-HSD1 inhibition decreases p-IRS-1^{Ser307} and increases p-AKT/p-PKB^{Thr308} [53,54]. Nevertheless, hepatic mRNA expression and/or activity of 11β-HSD1 and GR are dynamically regulated by the different disease's states, with 11β-HSD1 activity being decreased in simple steatosis but increased in NASH, with a similar variation for GR mRNA expression in NASH [3]. Surprisingly, and in contrast with the work by

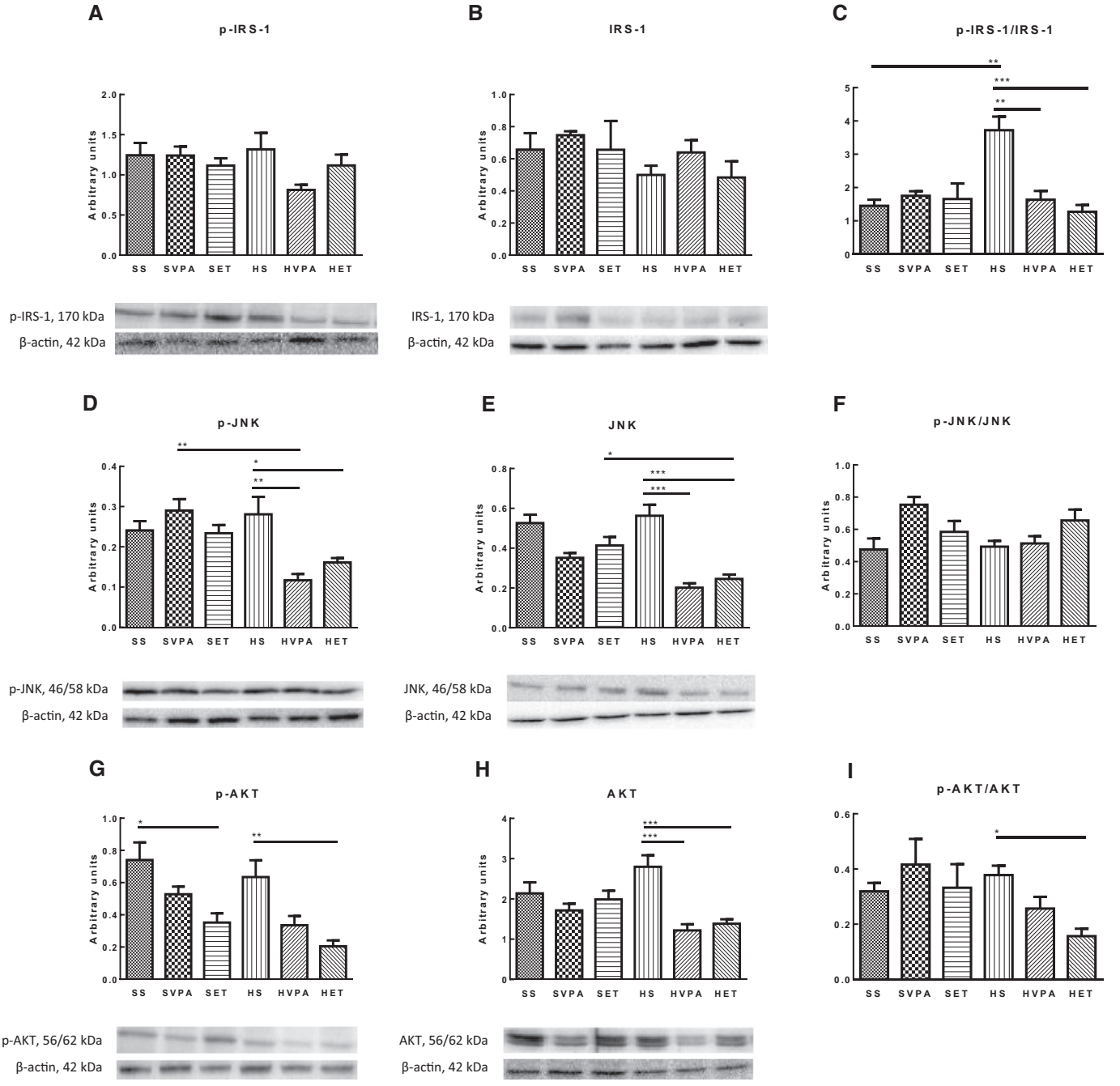


Fig. 4. The effect of diet, voluntary physical activity and endurance training on hepatic insulin signaling and stress parameters. Representative bands obtained by semiquantitative Western blot analysis of A) phosphorylated insulin receptor substrate-1 (p-IRS-1), B) total insulin receptor substrate-1 (IRS-1), D) phosphorylated c-Jun N-terminal protein kinase (p-JNK), E) total c-Jun N-terminal protein kinase (JNK), G) phosphorylated protein kinase B (p-AKT), H) total protein kinase B (AKT), J) phosphorylated extracellular signal-regulated kinase (p-ERK) and K) total extracellular signal-regulated kinase (ERK). Ratios of phosphorylated to total forms are also shown. C) p-IRS-1/IRS-1, F) p-JNK/JNK, I) p-AKT/AKT and L) p-ERK/ERK. Values are expressed as mean ± SEM. HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; kDa, kiloDalton; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

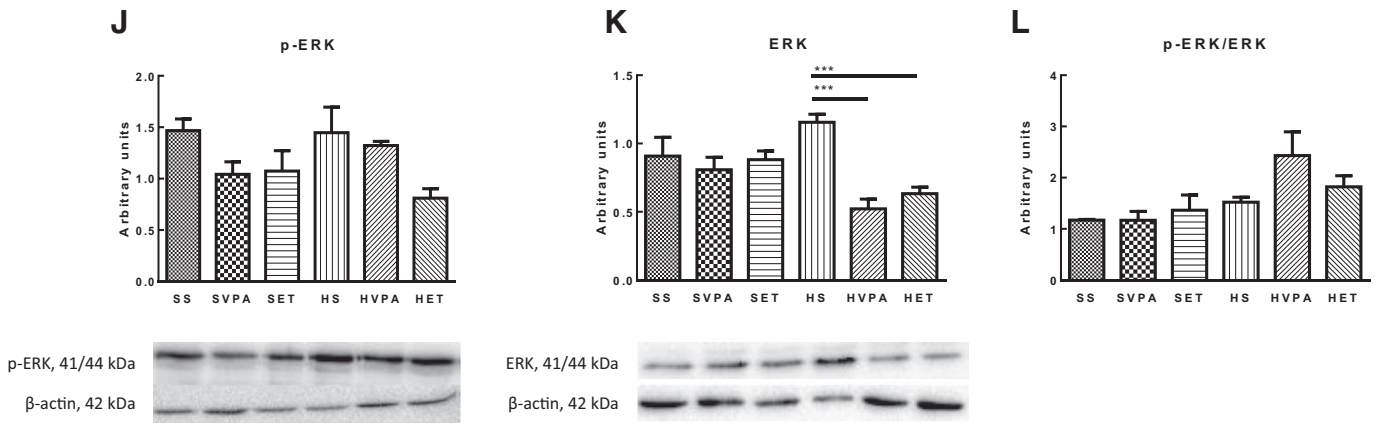


Fig. 4 (*continued*).

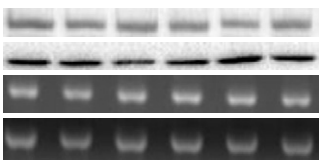
Coutinho and coworkers [56], an exercise-induced increased GR protein expression was also found in the present study. Hypothetically, an adaptive mechanism to counteract the reduction of the 11β-HSD1 protein expression induced by exercise may explain this result. So, further studies are warranted in order to clarify the possible adaptive mechanisms involved in glucocorticoid signaling regulation. Furthermore, it has been suggested that glucocorticoid capability to enhance UCP3 gene expression in the muscle can be repressed by Sirt1, since it impairs p300 association with GR, which is crucial for GR action [19]. In fact, Sirt1 has protective effects against hepatic steatosis, insulin resistance and diabetes [57–59]. Additionally, Sirt1 has been suggested to be an effective suppressor of inflammation by decreasing the hepatic mRNA expression of IL-1β, IL-6 and TNF-α in rodents in the context of HFD, probably through inhibition of the NF-κB transcriptional activity [59]. Sirt1 associates physically with RelA/p65 and inhibits transcription through direct deacetylation of RelA/p65 at lysine 310 [60]. Even though physical exercise decreased hepatic inflammation and 11β-HSD1 protein expression, both HFD and physical exercise showed no significant effects in Sirt1 hepatic protein expression in our animal model. Interestingly, Sirt1 overexpression (in C2C12 myotubes) may decrease the transcription and/or protein expression of PTP1B which may be associated with beneficial effects on insulin signaling [61]. However, in the present study, other mechanisms, rather than those involving this deacetylase, may have contributed to the improved insulin signaling found in HVPA and HET groups (*vs.* HS), as we did not find any differences in Sirt1 expression.

The reduction in PTP1B mRNA expression observed in HET animals can be explained, at least in part, by the general effect of physical exercise in the decrease of inflammation. Increased PTP1B expression has been observed in the liver of HFD-fed mice [14] and of NASH-patients [13], which could be associated with hepatic inflammation in the context of NASH as TNF-α induces increased hepatic mRNA and protein expression of PTP1B via nuclear NF-κB activation [14]. Liver-specific re-expression of PTP1B in mice lacking PTP1B resulted in the reduction of hepatic insulin sensitivity [16]. In our study, the reduction of PTP1B mRNA expression was associated with the reduction of 11β-HSD1 protein expression and reinforced the enhanced ISI observed in animals submitted to ET. Therefore, blocking or reducing the expression of PTP1B could be one mechanistic target of ET against hepatic insulin resistance.

Since we conducted a long-term (17 weeks) feeding program, it is possible that the activation of NF-κB achieved a new state of homeostasis, which could explain the lack of alteration of both IKK and NF-κB protein levels observed here. Despite the lack of activation of the IKK–NF-κB pathway, our results showed that HFD-feeding significantly increased IL-1β (with a similar pattern being observed for IL-6 and TGF-β protein expression). Both VPA and ET reduced those pro-inflammatory mediators most particularly in the HFD branch, which is in accordance with VPA and ET-induced decreased expression and activation of JNK [a well-known inducer of the transcription of pro-inflammatory mediators] in the HFD-fed rat liver.

Both inflammation and insulin resistance could represent possible links between NAFLD/NASH (the hepatic manifestation of the metabolic syndrome) and cardiovascular diseases, with JNK being a key player in this process [12,62]. In fact, JNK, which is activated by ER stress, in addition to its role in modulating inflammation and insulin resistance, appears to modulate the calcium flow between the ER and the mitochondria by regulating the activation of the pro-apoptotic bcl-2-like protein 4 (Bax), which binds and antagonizes the Bcl-2 protein [62]. While the serum concentration of the anti-apoptotic protein Bcl-2 appears to be lower in NASH patients compared with both simple fatty liver and healthy control

Table 2
The effect of diet, voluntary physical activity and endurance training on hepatic insulin signaling parameters. Representative bands obtained by semiquantitative Western blot analysis of the insulin receptor substrate 2 (IRS-2) protein and by reverse transcriptase PCR of the protein tyrosine phosphatase 1B (PTP1B) mRNA.

	SS	SVPA	SET	HS	HVPA	HET		
IRS-2	1.724 ± 0.28	1.306 ± 0.17	1.372 ± 0.16	1.240 ± 0.06	1.220 ± 0.17	1.542 ± 0.14		IRS-2, 170 kDa β-Actin, 42 kDa
PTP1B	1.764 ± 0.13	1.623 ± 0.01	1.603 ± 0.02	1.999 ± 0.12	1.983 ± 0.07	1.239 ± 0.20*		PTP1B, 408 bp GAPDH, 683 bp

Values are expressed as mean ± SEM.
bp, base pairs; HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; kDa, kiloDalton; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet. *$p < 0.05$ *vs.* HS and HVPA.

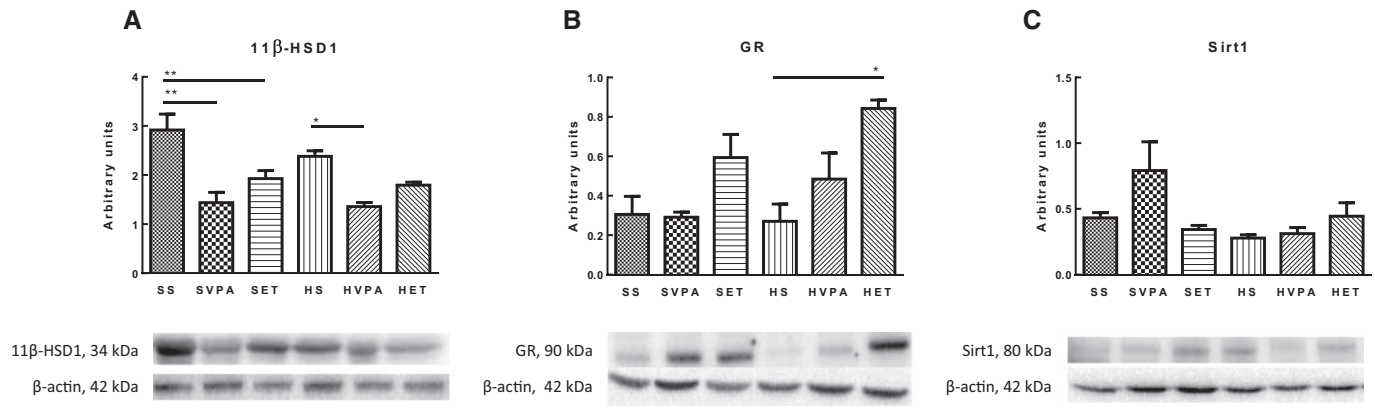


Fig. 5. The effect of diet, voluntary physical activity and endurance training on hepatic glucocorticoid signaling parameters. Representative bands obtained by semiquantitative Western blot analysis of A) 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), B) glucocorticoid receptor (GR) and C) sirtuin 1 (Sirt1). Values are expressed as mean ± SEM. HET, endurance trained animals with access to high-fat liquid diet; HS, sedentary animals with access to high-fat liquid diet; HVPA, voluntarily physically active animals with access to high-fat liquid diet; kDa, kiloDalton; SET, endurance trained animals with access to standard control liquid diet; SS, sedentary animals with access to standard control liquid diet; SVPA, voluntarily physically active animals with access to standard control liquid diet. $^{*}p < 0.05$ and $^{**}p < 0.01$.

subjects, hepatocyte apoptosis seems to be higher, which could imply an imbalance between pro-apoptotic and anti-apoptotic processes probably mediated by JNK [63,64].

Like IL-1β and IL-6, LIF (that belongs to the IL-6 super family) plays an important role in the context of inflammation, such as stimulation of the acute-phase response [65,66], and has been suggested to induce insulin resistance in isolated cardiomyocytes after chronic exposure [67]. However, so far, there is no study regarding the impact of exercise training on the modulation of the hepatic expression of LIF in the context of NASH. Here, we have found that VPA and ET (more evident in ET) decreased the hepatic expression of LIF in the HFD branch.

Pro-inflammatory cytokines activate ERK [10,65]. Additionally, increased ERK expression and/or activity is involved in the hepatic desensitization of the insulin signaling through inhibitory phosphorylation of IRS-1/2 [10,68]. In HFD rats, both VPA and ET modulated ERK signaling probably in order to protect insulin signaling, which seems to be consistent with the reduction of pro-inflammatory mediators, JNK signaling and the ratios of p-IRS-1/total IRS-1 and p-AKT/total AKT.

Although several other studies demonstrated that ET reduces the hepatic expression of pro-inflammatory mediators [28,36] and attenuates liver fibrosis [36], to our knowledge, this is the first study demonstrating the reduction of TGF-β hepatic expression by both VPA and ET, in an animal model of HFD-induced NASH. This effect could translate into a reduced risk of hepatic fibrosis and inflammation and, consequently, an improvement of insulin signaling. In accordance with other studies demonstrating that inhibition and/or down-regulation of TGF-β signaling results in decreased hepatic inflammation [4,8,25], here we observed that physical exercise-induced decrease of hepatic TGF-β associated with down-regulation of hepatic pro-inflammatory mediators. Therefore, we propose that the reduction of the pro-inflammatory state induced by exercise could be explained in part by the reduction of JNK pathway activation and TGF-β protein expression. Additionally, the reduction of the amount of adipose tissue observed in the animals submitted to physical exercise may have also contributed, as it has been suggested that liver inflammation in NASH originates in visceral adipose tissue and/or could be a response of adipose tissue-induced hepatic lipotoxicity [14,69]. In fact, we recently showed that sedentary HFD animals presented an increase of mesenteric and perirenal adipose tissues compared with standard diet-fed animals, which was completely mitigated by physical exercise (more strongly by ET) [30]. Therefore, it is plausible that physical exercise reduced free fatty acid and pro-inflammatory molecule delivery to the liver, drained from the visceral depot directly into the portal circulation.

Unfortunately, our study presents some limitations. The animal model of NAFLD/NASH shares some mechanisms, but not all, with the human illness. Secondly, like with every other animal models of NASH, exporting the mechanisms from an animal model to humans helps in understanding the mechanism underlying the pathophysiology of the disease but the translation should be done with caution. Finally, and considering the overall accordance of our results, a higher number of animals per group would have strengthened our conclusion.

Conclusion

Taken together, our data supports the idea that physical exercise could be an effective non-pharmacological preventive and therapeutic tool (namely VPA and ET, respectively) against NASH-associated features by modulating the hepatic insulin, pro-inflammatory and glucocorticoid signaling pathways as well as pro-inflammatory mediators. Although both physical exercise regimens seemed beneficial in counteracting the deleterious effects of NASH, only ET was able to improve ISI and significantly reduce both AKT activation and the ratio of phosphorylated AKT to total AKT. Additionally, ET appeared to be more efficient in reducing the hepatic inflammatory signals (IL-1β, IL-6 and LIF), reinforcing the major role of exercise intensity against NASH features.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

EP, AA, JM and MJM were responsible for the study concept and design. EP, IOG and SR-R took care of the animals' treatment and exercise protocols. EP performed most of the liver biochemical assays. CP and DN performed some Western blots. NS and JTG were responsible for the plasma biochemical analyses. EP, JM and MJM drafted the manuscript. All other co-authors critically revised the manuscript.

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RESEARCH ARTICLE

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Physical exercise positively modulates nonalcoholic steatohepatitis-related hepatic endoplasmic reticulum stress

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Abstract

Obesity is a predictive factor for the development of nonalcoholic steatohepatitis (NASH). Although some of the mechanisms associated with NASH development are still elusive, its pathogenesis relies on a complex broad spectrum of (interconnected) metabolic-based disorders. We analyzed the

Abbreviations: ATF-4, activating transcription factor-4; BiP, immunoglobulin heavy chain-binding protein homolog; BSA, bovine serum albumin; caspase, cysteine-dependent aspartate specific protease; CHOP, C/EPB homologous protein; eIF2α, eukaryotic initiation factor-2 alpha; ER, endoplasmic reticulum; ET, endurance training; GADD34, growth arrest and DNA damage-inducible protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-S-transferase; GSX, total glutathione; HFD, high-fat diet; HS, sedentary animals with access to liquid HFD; HT, endurance-trained animals with access to liquid HFD; HV, voluntarily physically active animals with access to liquid HFD; IRE1α, inositol requiring enzyme-1 alpha; JNK, c-Jun *N*-terminal kinase; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; Nrf2, nuclear erythroid 2 p45-related factor; PCR, polymerase chain reaction; PE, physical exercise; p-eIF2α, phosphorylated eukaryotic initiation factor-2 alpha; PERK, protein kinase RNA-activated (PKR)-like ER kinase; p-IRE1α, phosphorylated inositol requiring enzyme-1 alpha; p-PERK, phosphorylated protein kinase RNA-activated (PKR)-like ER kinase; RIDD, regulated IRE1-dependent decay; RT, reverse transcriptase; RT-PCR, reverse transcriptase-polymerase chain reaction; SCLD, standard control liquid diet; SOD, superoxide dismutase; SS, sedentary animals with access to SCLD; ST, endurance-trained animals with access to SCLD; SV, voluntarily physically active animals with access to SCLD; sXBP1, spliced X-box binding protein 1; TBARS, thiobarbituric acid reactive substances; TBST, Tris-base-buffered saline with 0.1% (vol/vol) Tween-20; TRAF2, tumor necrosis factor receptor-associated factor 2; UPR, unfolded protein response; uXBP1, unspliced X-box binding protein 1; VPA, voluntary physical activity; WB, Western blot; XBP1, X-box binding protein 1.

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effects of voluntary physical activity (VPA) and endurance training (ET), as preventive and therapeutic nonpharmacological strategies, respectively, against hepatic endoplasmic reticulum (ER) stress, ER-related proapoptotic signaling, and oxidative stress in an animal model of high-fat diet (HFD)-induced NASH. Adult male Sprague–Dawley rats were divided into standard control liquid diet (SCLD) or HFD groups, with sedentary, VPA, and ET subgroups in both (sedentary animals with access to SCLD [SS], voluntarily physically active animals with access to SCLD [SV], and endurance-trained animals with access to SCLD [ST] in the former and sedentary animals with access to liquid HFD [HS], voluntarily physically active animals with access to liquid HFD [HV], and endurance-trained animals with access to liquid HFD [HT] in the latter, respectively). Hepatic ER stress and ER-related proapoptotic signaling were evaluated by Western blot and reverse transcriptase-polymerase chain reaction; redox status was evaluated through quantification of lipid peroxidation, protein carbonyls groups, and glutathione levels as well as antioxidant enzymes activity. In SCLD-treated animals, VPA significantly decreased eukaryotic initiation factor-2 alpha (eIF2α). In HFD-treated animals, VPA significantly decreased eIF2α and phospho-inositol requiring enzyme-1 alpha (IRE1α) but ET significantly decreased eIF2α and significantly increased both spliced X-box binding protein 1 (sXBP1) and unspliced X-box binding protein 1; a significant increase of phosphorylated-eIF2α (p-eIF2α) to eIF2α ratio occurred in ET versus VPA. HS compared to SS disclosed a significant increase of total and reduced glutathione, HV compared to SV a significant increase of oxidized glutathione, HT compared to ST a significant increase of p-eIF2α to eIF2α ratio and sXBP1. Physical exercise counteracts NASH-related ER stress and its associated deleterious consequences through a positive and dynamical modulation of the hepatic IRE1α–X-box binding protein 1 pathway.

KEYWORDS
cell death, nonalcoholic steatohepatitis, oxidative stress, physical exercise, unfolded protein response

1 | INTRODUCTION

Nowadays, due to a sedentary lifestyle and excessive consumption of high-calorie foods, the world faces a dramatic increase in chronic (interconnected) metabolic disturbances, of which obesity, insulin resistance, and type 2 diabetes mellitus are widespread examples.

Obesity induction of ectopic hepatic lipid droplet accumulation can result in nonalcoholic fatty liver disease (NAFLD), which includes a progression from steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and, eventually, hepatocellular carcinoma.[2,4–6] In fact, NAFLD is currently recognized as a novel worldwide epidemic and is the most common form of liver disease affecting between 25% and 32% of the general population.[7–10] NASH is strongly associated with features of metabolic syndrome and increased risk of cardiovascular complications,[7,10–12] NASH patients exhibit an increased liver-related mortality due to the progression to cirrhosis and hepatocellular carcinoma.[10,13]

Besides the critical role played by the lipotoxicity of the aberrant hepatic lipid accumulation, several other factors have been implicated in the pathogenesis of NASH triggered by obesity, namely disturbed fatty acid and cholesterol metabolism, increased endoplasmic reticulum (ER) and oxidative stresses, decreased mitochondrial function,[6,7,14–17] insulin resistance,[6,15,18] altered tissue

lipid composition,[19–21] increased inflammation,[22,23] and increased apoptotic signaling.[6,24] Additionally, being the ER an important sensor of cellular stress,[22,23,25,26] the hepatic ER dysfunction associated with NASH excessive cellular requirements is considered a key player of the abovementioned interrelated deleterious processes in this highly metabolic tissue.[27] In fact, under pathological conditions, ER can play a major role in the amplification of a harmful vicious cycle, as delayed or inadequate ER stress responses *per se* can exacerbate fat accumulation, insulin resistance, oxidative stress, mitochondrial dysfunction, proinflammatory processes, and apoptosis.[1,2,6,27,28] For example, being the ER and mitochondria physically and functionally interconnected, changes in the ER redox balance influences mitochondrial function by enhancing mitochondrial oxidative stress.[4,29] Moreover, mitochondrial reactive oxygen species increase proinflammatory signaling, concomitantly leading to insulin signaling dysfunction.[4,6,27,29]

However, despite the increasing number of scientific efforts,[3,9,13,29] the identification and management of an effective pharmacological therapy for NASH treatment is still lacking. Interestingly, physical exercise (PE), used as nonpharmacological preventive and/or therapeutic strategy in several metabolic dysfunctions, attenuates hepatic lipid accumulation[5] and restores mitochondrial morphology, structural network, bioenergetics, and metabolism[30,31] as well as mitigates the expression of proinflammatory mediators and improves insulin signaling[18] in an animal model of high-fat diet (HFD)-induced NASH. In addition, PE also seems to be effective against NASH-related ER stress itself.[27] Nevertheless, the full extent of mechanisms by which PE mitigates the development and progression of NASH-related ER stress is not yet completely unraveled neither their interconnection fully understood.

Therefore, considering the orchestrated plethora of interdependent factors involved in the hepatic dysfunction associated with NASH, the present study aimed to further identify the mechanisms by which PE attenuates the aforementioned ER-induced vicious cycle and protects the liver against ER and oxidative stresses and ER-related proapoptotic signaling in an animal model of HFD-induced NASH.

2 | MATERIALS AND METHODS

2.1 | Animal treatment

Thirty-six male Sprague–Dawley rats (aged 5–6 weeks and weighing 125–150 g) were purchased from Charles River. Animals were individually housed in cages (with an enriched environment), with *ad libitum* access to water and food (provided in the liquid state, purchased from Dyets Inc.) and maintained in a temperature and humidity-controlled room (21–22°C; 50%–60% humidity), with reversed 12 h light/dark cycles.

Before the beginning of the dietary and activity protocols, animals went through an adaptation process to the liquid diet (1 week), in which a standard control liquid diet (SCLD) was given to all animals. Then, the animals have randomly ascribed to six groups as follows: SCLD + sedentary animals (SS), SCLD + voluntarily physically active animals (SV), SCLD + endurance-trained animals (ST), liquid HFD + sedentary animals (HS), liquid HFD + voluntarily physically active animals (HV), and liquid HFD + endurance-trained animals (HT).

Both diets were purchased from Dyets Inc. The liquid HFD (previously described to induce NASH[31]) provided 71% of energy from fats, 11% from carbohydrates, and 18% from proteins (Lieber–DeCarli diet #712031) while the isocaloric SCLD provided 35% of energy from fats, 47% from carbohydrates, and 18% from proteins (Lieber–DeCarli diet #710027).

Animals of the SV and HV groups had free access to a freewheel throughout the 17 weeks of the protocol (Figure 1). The running distance was obtained daily, between 8:00 and 10:00 a.m., from a digital counter. After 8 weeks of diet consumption, half of the SS and HS animals were submitted to endurance training (ET) (ST and HT, respectively), while the other half continued to be sedentary. The training regimen was preceded by an adaptation process, in which ST and HT rats were progressively acclimatized to the motor-driven treadmill for 5 days, at 15 m/min and 0% grade, until 30 min of running was achieved. This 1-week adaptation process was followed by 8 weeks of endurance exercise for 5 days/week, 60 min/day, at a starting speed of 15 m/min, which was gradually increased over the training program until the speed of 25 m/min was reached. To expose the sedentary animals (SS and HS) to the same environmental conditions, but without promoting any physical training adaptation, sedentary animals were placed on a nonmoving treadmill 5 days/week, for 60 min/day.

The study was approved by the local Institutional Ethics Committee and followed the guidelines for the care and use of laboratory animals in research advised by the Federation of European Laboratory Animal Science Associations (FELASA) and Portuguese Act 129/92. Several authors of this manuscript are accredited by FELASA to perform animal experimentation.

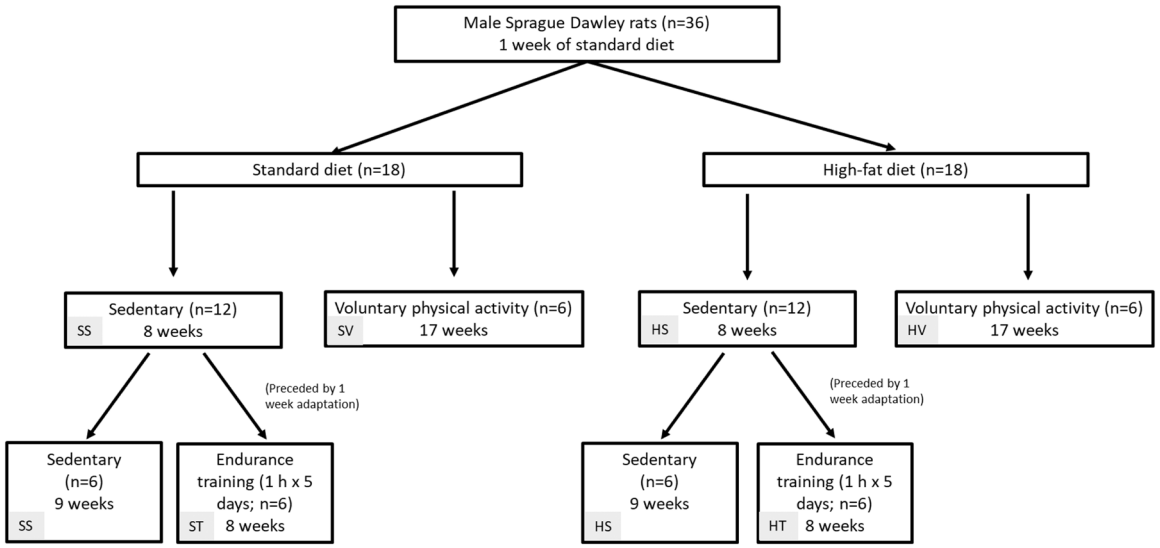

FIGURE 1 Design of the dietary and exercise training protocols. HS, sedentary animals with access to liquid high-fat diet; HT, endurance-trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; SS, sedentary animals with access to standard control liquid diet; ST, endurance-trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet

2.2 | Collection of liver samples

Rats were deeply anesthetized with a ketamine/xylazine combination and transcardiacally perfused with an ice-cold isotonic sodium chloride solution. After perfusion, the liver was rapidly removed from the abdominal cavity, washed in the same solution, dried in qualitative paper, weighted, and cut into several fragments. Liver fragments were either placed in a 10% buffered formalin solution for histological analysis or immediately stored and kept at −80°C until enzymatic, spectrophotometric, reverse transcriptase (RT) polymerase chain reaction (RT-PCR), and immune-blotting (Western blot, WB) analysis were performed.

2.3 | Histological analysis

Liver fragments placed in a 10% buffered formalin solution, were progressively dehydrated in increasing concentrations of ethanol (70%–100%), and embedded in paraffin. Then, the samples were sectioned (5 μm; three sections per slide), deparaffinized with xylene, and stained with hematoxylin and eosin. After these proceedings, samples were blindly and randomly examined under light microscopy (Olympus BX61) and classified according to the NAFLD activity score (NAS). The NAS score was achieved by summing up the scores of steatosis (0–3), lobular inflammation (0–2), and hepatocellular ballooning (0–2). A total score of 0–2 represents no steatohepatitis, 3–4 reflects questionable steatohepatitis, and 5 or more mirrors definite steatohepatitis.[32]

2.4 | RNA extraction

Liver total RNA was extracted using Tripure® Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics). Then, pellets were dissolved in diethylpyrocarbonate-treated water and stored at −80°C, until further used for RT-PCR. The quantity and quality of isolated RNA were assessed by reading the absorbance at 230, 260, and 280 nm, using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific).

2.5 | Reverse transcriptase-polymerase chain reaction

Samples with ratios of A260/A280 > 1.9 were used for complementary DNA (cDNA) synthesis. Two-point 5 mg of total RNA were used as a template for cDNA production using the NZY First-strand cDNA Synthesis Kit (NZYTech Lda; https://www.nzytech.com/site/cDNA-Synthesis-kits/NZY-First-Strand-cDNA-Synthesis-Kit), as described before.[18] The RT product was stored at −20°C until required.

Using 2 μL of RT product, the RT-PCR amplification of a given target gene and the invariant housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were simultaneously performed. Primer sequences,

expected RT-PCR product sizes, and RT-PCR conditions are depicted in Table 1.

Individual RT-PCR reaction products were visualized on 2.5% (vol/vol) agarose gel (except for X-box binding protein 1 [XBP1] which was visualized on 3.5% [vol/vol] agarose gel) with ethidium bromide staining (0.4 μg/mL), using the Gel Doc-It™ TS Imaging System (UVP). The expression of each gene was determined using the Visionworks LS® Software (UVP) and normalized to the GAPDH value of the same sample. The expression of this enzyme was not modified by the dietary protocol (*data not shown*). The best set of six bands (SS, SV, ST, HS, HV, and HT animals)/messenger RNA (mRNA), matching the mean of each group, was selected for the corresponding figures.

2.6 | Protein extraction and Western blot

Total protein was extracted from liver fragments as described in Passos et al.,[18] and kept at −80°C until further analysis. The protein content in each extract was assessed using the Bradford method.[33]

Fifty milligrams of each sample were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (ranging from 6% to 12%), as described by Laemmli,[34] followed by blotting into a nitrocellulose membrane (Hybond; Amersham GE Healthcare) according to the method of Locke et al.[35] Then, the membranes were stained with *Ponceau S* to evaluate the quality of the protein transfer. Membranes were washed with Tris-base-buffered saline with 0.1% (vol/vol) Tween-20 (TBST) to remove the *Ponceau S* staining. Nonspecific binding was blocked with TBST containing 2.5%–5% (vol/vol) nonfat dry powdered milk (Bio-Rad Laboratories Inc.) or 2.5%–5% (vol/vol) bovine serum albumin (BSA; NZYTech Lda) in TBST for 1 h, at room temperature. Then, the membranes were incubated with the primary antibody, with gentle agitation, overnight at 4°C, diluted in 2.5%–5% (vol/vol) nonfat dry milk or 2.5%–5% (vol/vol) BSA in TBST. Subsequently to this incubation, the membranes were washed in TBST and incubated with the secondary antibody conjugated to horseradish peroxidase, for 1 h at room temperature. All membranes were stripped and re-probed with a β-actin primary antibody as described above (Table 2). Protein band levels were a) quantified after enhanced chemiluminescence staining using the ChemiDoc™ XRS+ System (Bio-Rad Laboratories Inc.) and the ImageLab™ software (version 4.0.1, Bio-Rad Laboratories Inc.) and b) normalized for β-actin expression. The expression of this enzyme was not modified by the dietary protocol (*data not shown*). The best set of six bands (SS, SV, ST, HS, HV, and HT animals)/protein, matching the mean of each group, was selected for the corresponding figures.

TABLE 1 Reverse transcriptase-polymerase chain reaction: primer sequences, expected products size, and experimental conditions

Gene	Primer sequence (5′–3′)	Denaturation	Annealing	Elongation	Number of cycles
ATF-4 (404 bp)	F – TCCTGAACAGCGAAGTGTTG R – GCCAATTGGGTTCACTGTCT	94°C, 45 s	60°C, 45 s	72°C, 45 s	25
ATF-6 (345 bp)	F – CAGGTGAAGACTGGGAGTCCACGT R – TGTGACAGCTGTCGCCATACAAG	94°C, 45 s	60°C, 45 s	72°C, 45 s	30
BiP (213 bp)	F – GCGGAGGAGGAGGACAAGAA R – CGGGTTGGACGTGAGTTGGT	94°C, 45 s	58°C, 45 s	72°C, 45 s	30
CHOP (383 bp)	F – TACCATGTTGAAGATGAGCGGG R – TCTGGAGAGCGAGGGCTTTG	94°C, 45 s	59°C, 45 s	72°C, 45 s	30
GADD34 (294 bp)	F – AAGCGGCTCAGATCTTTCAA R – AGACGAGGACGACAGAGGAA	94°C, 45 s	57°C, 45 s	72°C, 45 s	30
XBP1 (289 bp)	F – TTACGAGAGAAAACTCATGGGC R – GGGTCCAACTTGTCCAGAATGC	94°C, 45 s	58°C, 45 s	72°C, 45 s	30
GAPDH (683 bp)	F – ACTGGCGTCTTCACCACCAT R – TCCACCACCCTGTTGCTGTA				25 or 30

Abbreviations: ATF-4, activating transcription factor-4; ATF-6, activating transcription factor-6; BiP, immunoglobulin heavy chain-binding protein homolog; bp, base pairs; CHOP, C/EPB homologous protein; F, forward; GADD34, growth arrest and DNA damage-inducible protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; R, reverse; XBP1, X-box binding protein 1.

TABLE 2 Western blot conditions details

Protein	Primary antibody incubation	Primary antibody brands	Secondary antibody incubation	Secondary antibody brands
Caspase-12	1:500 in 5% BSA, 1 overnight, at 4°C	ab18766 (rabbit polyclonal antibody); Abcam	1:500 in 5% blotto, 1 h at RT	
eIF2α	1:200 in 5% blotto, 1 overnight, at 4°C	sc-30882 (goat polyclonal antibody); Santa Cruz Biotechnology	1:500 in 5% blotto, 1 h at RT	
p-eIF2α	1:200 in 5% BSA, 1 overnight, at 4°C	Ser 52; sc-12412 (goat polyclonal antibody); Santa Cruz Biotechnology	1:500 in 5% blotto, 1 h at RT	
IRE1α	1:500 in 1% blotto, 1 overnight, at 4°C	sc-20790 (rabbit polyclonal antibody); Santa Cruz Biotechnology	1:500 in 1% blotto, 1 h at RT	
p-IRE1α	1:500 in 1% blotto, 1 overnight, at 4°C	54570 (rabbit polyclonal antibody); AnaSpec Inc.	1:500 in 1% blotto, 1 h at RT	
PERK	1:750 in 5% blotto, 1 overnight, at 4°C	sc-13073 (rabbit polyclonal antibody); Santa Cruz Biotechnology	1:500 in 5% blotto, 1 h at RT	
p-PERK	1:200 in 5% BSA, 1 overnight, at 4°C	Thr 981; sc-32577 (rabbit polyclonal antibody); Santa Cruz Biotechnology	1:500 in 5% blotto, 1 h at RT	
β-actin	1:1000 in 5% blotto, 1 overnight, at 4°C	sc-1616 (goat polyclonal antibody); Santa Cruz Biotechnology	1:2000 in 5% blotto, 1 h at RT	
Mouse anti-goat				sc-2354; Santa Cruz Biotechnology
Goat anti-mouse				sc-2005; Santa Cruz Biotechnology
Goat anti-rabbit				sc-2004; Santa Cruz Biotechnology

Abbreviations: Blotto, nonfat dry powdered milk diluted in Tris-base-buffered saline with 0.1% (vol/vol) Tween-20; BSA, bovine serum albumin; caspase-12, cysteine-dependent aspartate specific protease 12; eIF2α, total eukaryotic initiation factor-2 alpha; ER, endoplasmic reticulum; IRE1α, total inositol requiring enzyme-1 alpha; p-eIF2α, phosphorylated eukaryotic initiation factor-2 alpha; PERK, total protein kinase RNA-activated (PKR)-like ER kinase; p-IRE1α, phosphorylated inositol requiring enzyme-1 alpha; p-PERK, phosphorylated protein kinase RNA-activated (PKR)-like ER kinase; RT, room temperature.

2.7 | Assessment of ER stress signaling parameters

ER stress was evaluated by both mRNA and protein expression. Immunoglobulin heavy chain-binding protein homolog (BiP), activating transcription factor (ATF)-4 and ATF-6, and unspliced (u)XBP1, besides spliced (s) XBP1, were evaluated by RT-PCR (as previously described). RT-PCR details are listed in Table 1. Phosphorylated protein kinase RNA-activated (PKR)-like ER kinase (p-PERK), total protein kinase RNA-activated (PKR)-like ER kinase (PERK), phosphorylated eukaryotic initiation factor-2 alpha (p-eIF2α), total eukaryotic initiation factor-2 alpha (eIF2α), phosphorylated inositol requiring enzyme-1 alpha (p-IRE1α), and total inositol requiring enzyme-1 alpha (IRE1α) were determined by WB (as previously described). WB conditions (including primary and secondary antibodies dilutions) are shown in Table 2.

2.8 | Assessment of redox status parameters

For the assessment of redox state markers, and subsequent characterization of oxidative stress level, a small portion of hepatic tissue was sliced and homogenized in cold phosphate buffer (pH 7.4), containing 0.1% Triton X-100, with a glass-Teflon homogenizer. Tissue homogenates were centrifuged (16 000 *g*, 10 min, 4°C) and the supernatants were separated to be used in the different biochemical assays of oxidative stress.

Oxidative damage was evaluated through the determination of lipid peroxidation and protein carbonyl groups levels by using the thiobarbituric acid reactive substances (TBARS) assay and the reaction with 2,4-dinitrophenylhydrazine, respectively. The appraisal of hepatic antioxidant defenses included the quantification of catalase, superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR) activities. Moreover, both total (GSX), reduced (GSH), and oxidized (GSSG) glutathione intracellular levels were determined. All these techniques are routinely used in our laboratory.[36–39]

2.9 | Assessment of ER stress-related proapoptotic signaling parameters

Hepatic activation of apoptotic signaling was evaluated by the quantification of a) growth arrest and DNA damage-inducible protein (GADD34) and C/EPB homologous protein (CHOP) mRNA expression, along with b) cysteine-dependent aspartate specific protease (caspase)-12 protein expression, as described before.

2.10 | Statistical analysis

Statistical analysis was performed with GraphPad Prism® software (GraphPad version 9.0.0). The statistical significance of the differences among the groups was evaluated using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Values are presented as mean ± standard error of the mean. Differences between experimental groups were considered significant with a confidence interval of 95%, whenever $p < 0.05$ (a strong tendency was considered whenever $0.05 < p \leq 0.1$).

3 | RESULTS

3.1 | Hepatic histological analysis

Both HS (Figure 2D) and HV (Figure 2E) groups developed a morphological phenotype consistent with a NASH pathological condition when compared to SS (Figure 2A) and SV (Figure 2B) groups, respectively ($p < 0.05$, Table 3). Interestingly, in HFD-fed animals, ET program engagement counteracted the histopathological features and score classification observed in their sedentary lifestyle counterparts (HT vs. HS, Figure 2F,D), being the NASH classification downgraded from "definitive NASH" to "questionable NASH."

3.2 | Hepatic ER stress signaling parameters

Neither PE regimens nor dietary intervention induced relevant effects upon p-PERK (Figure 3A) and total PERK (Figure 3B) protein expression or the ratio of p-PERK to total PERK (Figure 3C).

The protein expression of both p-eIF2α and total eIF2α presented a quite similar variation pattern (Figure 3D,E). For both SCLD and HFD, an identical variation pattern for p-eIF2α was found: voluntary physical activity (VPA) tended to decrease p-eIF2α protein expression when compared to the corresponding sedentary situation ($p = 0.058$ and $p = 0.082$, respectively; Figure 3D) while ET did not induce a relevant decreasing tendency pattern. Also, for both SCLD and HFD, an identical decreasing pattern with VPA, although significant ($p < 0.05$), was observed for total eIF2α protein expression, along with a similar significant decrease in

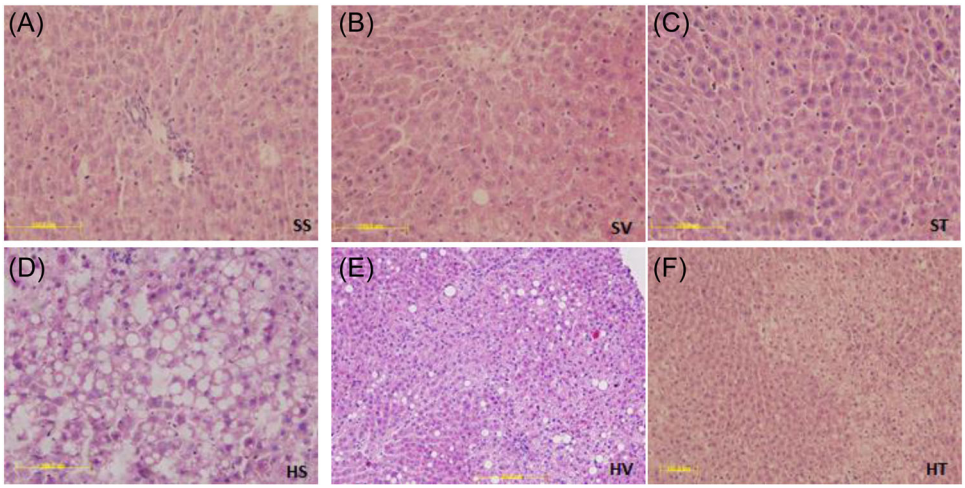

FIGURE 2 Histological image from livers of experimental groups under light microscopy. HS, sedentary animals with access to liquid high-fat diet; HT, endurance-trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; SS, sedentary animals with access to standard control liquid diet; ST, endurance-trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet.

TABLE 3 NAS score was recorded from the livers of all experimental groups, under light microscopy

Groups	SS	SV	ST	HS	HV	HT
NAS score	2.0 ± 1.0	2.5 ± 0.5	2.0 ± 1.1	6.1 ± 0.1^{a}	5.3 ± 0.8^{b}	3.5 ± 0.4^{c}

Note: NAS score, 0–2, no NASH; 3–4, questionable NASH; and ≥5, definitive NASH according to Kleiner et al.[32] Values are expressed as mean ± SEM. Different letter superscript indicates significant differences between groups in NAS score: [a]HS versus SS; [b]HV versus SV; and [c]HT versus HS. $p < 0.05$.

Abbreviations: HS, sedentary animals with access to liquid high-fat diet; HT, endurance-trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; SS, sedentary animals with access to standard control liquid diet; ST, endurance-trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet.

ET trained versus sedentary rats for HFD ($p = 0.01$, Figure 3E). The ratio of p-eIF2α to total eIF2α was significantly higher in HT than in the ST group ($p < 0.01$; Figure 3F), with a strong contribution from the fact that ST rats tended to show a higher total eIF2α protein expression than HT rats ($p = 0.108$; Figure 3E). In addition, this ratio was higher for ET than for VPA-trained animals in the HFD arm of the study ($p < 0.05$; Figure 3F).

Neither PE regimens nor dietary intervention led to significant effects on ATF-4 (Figure 3G), BiP (Figure 3H), and ATF-6 (Figure 3I) mRNA expression. Nevertheless, ATF-4 mRNA expression tended to be lower (Figure 3G) while ATF-6 mRNA expression tended to be higher (Figure 3I) in HT rats than in their SCLD counterparts ($p = 0.079$ and $p = 0.095$, respectively). Furthermore, ATF-6 mRNA expression tended to be higher in HT *versus*HS ($p = 0.083$; Figure 3I).

The protein expression of p-IRE1α revealed quite similar levels in the three SCLD groups (Figure 3J). HFD had no relevant effect on this ER stress marker (Figure 3J). Within the HFD arm of the study, a significant decrease was observed for p-IRE1 protein expression for HS *versus* HV ($p < 0.05$, Figure 3J), while was ET did not induce such an impact. No major alterations as a consequence of PE regimens or dietary intervention were observed either upon total IRE-1 protein expression (Figure 3K) or the ratio of p-IRE1α to total IRE1α (Figure 3L).

Regarding sXBP1 and uXBP1 mRNA expression, data showed a quite similar pattern of variation (Figures 3M and 3N, respectively), with identical levels for each mRNA on the three SCLD groups and significantly higher values in HT *versus* HS [$p < 0.05$ for both sXBP1 (Figure 3M) and uXBP1 (Figure 3N)]. In addition, HFD significantly increased sXBP1 in ET

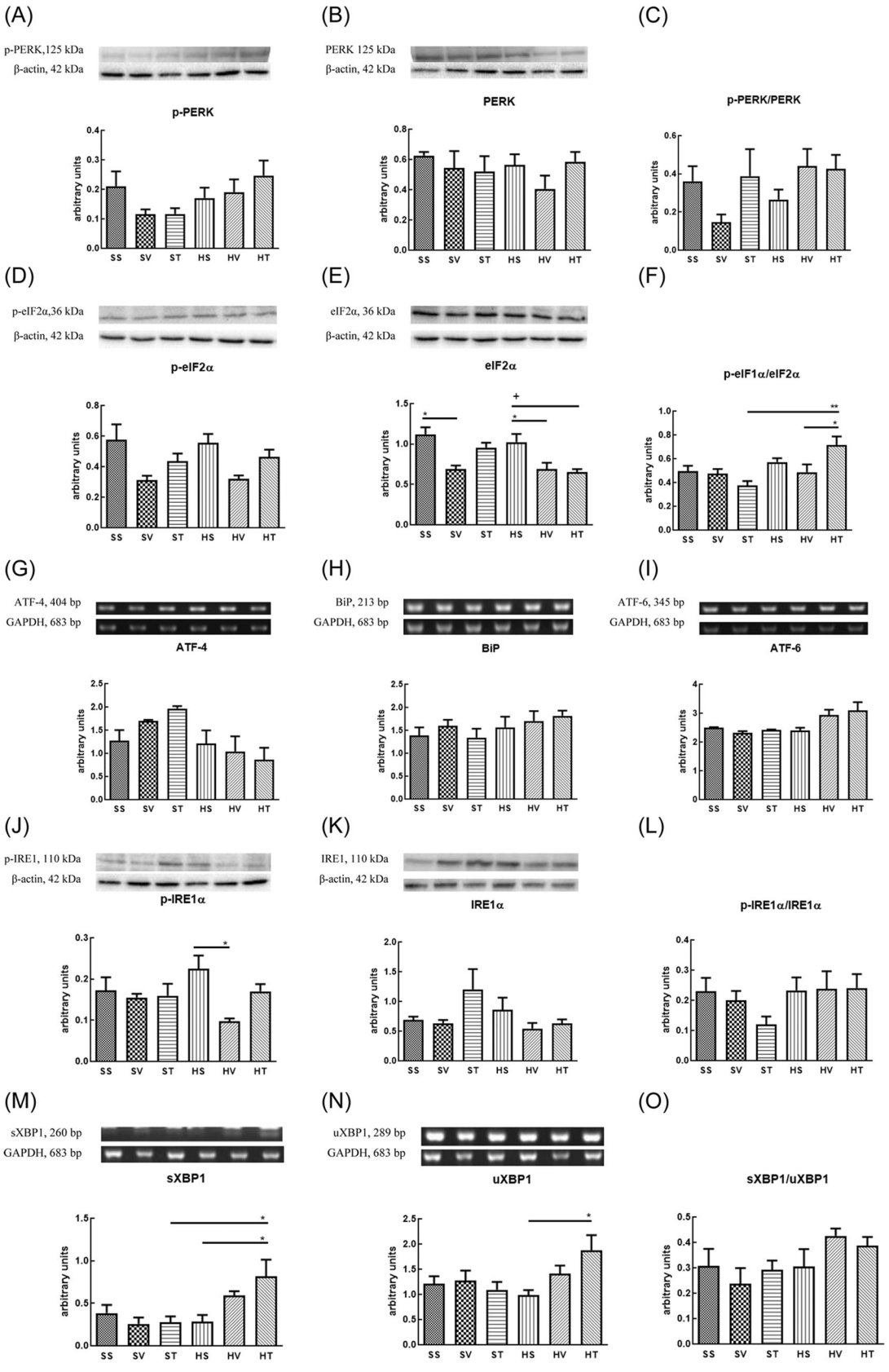

FIGURE 3 (See caption on next page)

animals ($p < 0.05$, Figure 3M), while uXBP1 showed a tendency for this outline ($p = 0.112$, Figure 3N). No crucial differences were observed in the ratio of sXPB1 to uXBP1 among the six groups of rats (Figure 3O).

3.3 | Hepatic redox status parameters

Neither PE regimens nor dietary intervention led to significant effects on malondialdehyde (Figure 4A) and protein carbonyl groups levels (Figure 4B) as well as catalase (Figure 4G), SOD (Figure 4H), GPx (Figure 4I), GR (Figure 4J), and GST (Figure 4K) activities. Catalase activity tended to be higher in SV versus SS ($p = 0.093$, Figure 4G).

The levels of GSX (Figure 4C) and GSH (Figure 4D) revealed an identical variation pattern, the sedentary HFD-fed animals with significantly higher values than sedentary SCLD-fed animals ($p < 0.001$ for GSX and $p < 0.01$ for GSH). Additionally, the level of GSX tended to be higher in HS when compared to HT ($p = 0.111$). The level of GSSG (Figure 4E) was significantly lower in SV when compared to HV ($p < 0.05$). No major differences were observed in the ratio of GSH to GSSG among groups (Figure 4F).

3.4 | Hepatic ER stress-related proapoptotic signaling parameters

Neither PE regimens nor dietary intervention led to important effects on CHOP and GADD34 mRNA expression (Figure 5A,B) or caspase-12 protein expression (Figure 5C). Considering the HFD-treated groups, GADD34 presented an outline of variation which can also be seen for caspase-12 (Figure 5B,C).

4 | DISCUSSION

NASH is a very serious health problem with deleterious consequences at several levels. In fact, NASH is not an isolated disease but it is strongly associated with metabolic syndrome, type 2 diabetes, and cardiovascular disease, with multiple pathways involved in this association.[13] Currently, several liver-targeted and other potential therapies are under investigation, pointing towards a broad range of pathologic changes associated with NASH, including insulin resistance, alterations in the microbiome and gut permeability, oxidative stress, apoptosis, lipotoxicity, inflammation, and altered bile acid metabolism.[13] However, besides PE regimens and dietary weight loss interventions, there are no pharmacological validated therapies for NASH treatment. In fact, there are substantial management gaps between clinical guidelines and clinical practice in what concerns NASH patients. In this regard, several outstanding medical associations initiated a "Call to Action" to issue guiding strategies for the management of NASH[8,13] due to its increasing impact on global public health and substantial socioeconomic burden.[10,40] Until that, PE appears to be the gold-standard preventive and/or therapeutic nonpharmacological strategy against several negative consequences of many obesity-associated metabolic dysfunctions.[5,14,18,41,42]

In accordance with the histological score from Kleiner et al.,[32] our results showed that sedentary animals fed with HFD have the typical hepatic injury induced by NASH in humans, such as steatosis, hepatocellular ballooning, and hepatic inflammation (as expected[43]). Here, we demonstrate that ET, but not VPA, was able to positively counteract the hepatic histological features induced by the HFD regimen. In our previous study,[18] we showed that PE could be an effective nonpharmacological preventive and therapeutic tool (namely VPA and ET, respectively) against NASH-associated features by modulating hepatic

FIGURE 3 Effects of diet, voluntary physical activity, and endurance training on hepatic endoplasmic reticulum (ER) stress signaling parameters. Representative bands were obtained by semiquantitative Western blot analysis or reverse transcriptase-polymerase chain reaction. (A) phosphorylated protein kinase RNA-activated (PKR)-like ER kinase (p-PERK); (B) total protein kinase RNA-activated (PKR)-like ER kinase (PERK); (C) ratio of p-PERK to PERK; (D) phosphorylated eukaryotic initiation factor-2 alpha (p-eIF2α); (E) total eukaryotic initiation factor-2 alpha (eIF2α); (F) ratio p-eIF2α to eIF2α; (G) activating transcription factor-4 (ATF-4); (H) immunoglobulin heavy chain-binding protein homolog (BiP); (I) activating transcription factor-6 (ATF-6); (J) phosphorylated inositol requiring enzyme-1 alpha (p-IRE1α); (K) total inositol requiring enzyme-1 alpha (IRE1α); (L) ratio of p-IRE1α to IRE1α); (M) spliced X-box binding protein 1 (sXBP1); (N) unspliced X-box binding protein 1 (uXBP1); and (O) ratio of sXBP1 to uXBP1. Values are expressed as mean ± SEM. bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HS, sedentary animals with access to liquid high-fat diet; HT, endurance-trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; kDa, kilodalton; SS, sedentary animals with access to standard control liquid diet; ST, endurance-trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet. *$p < 0.05$, $^{+}p = 0.01$, and **$p < 0.01$.

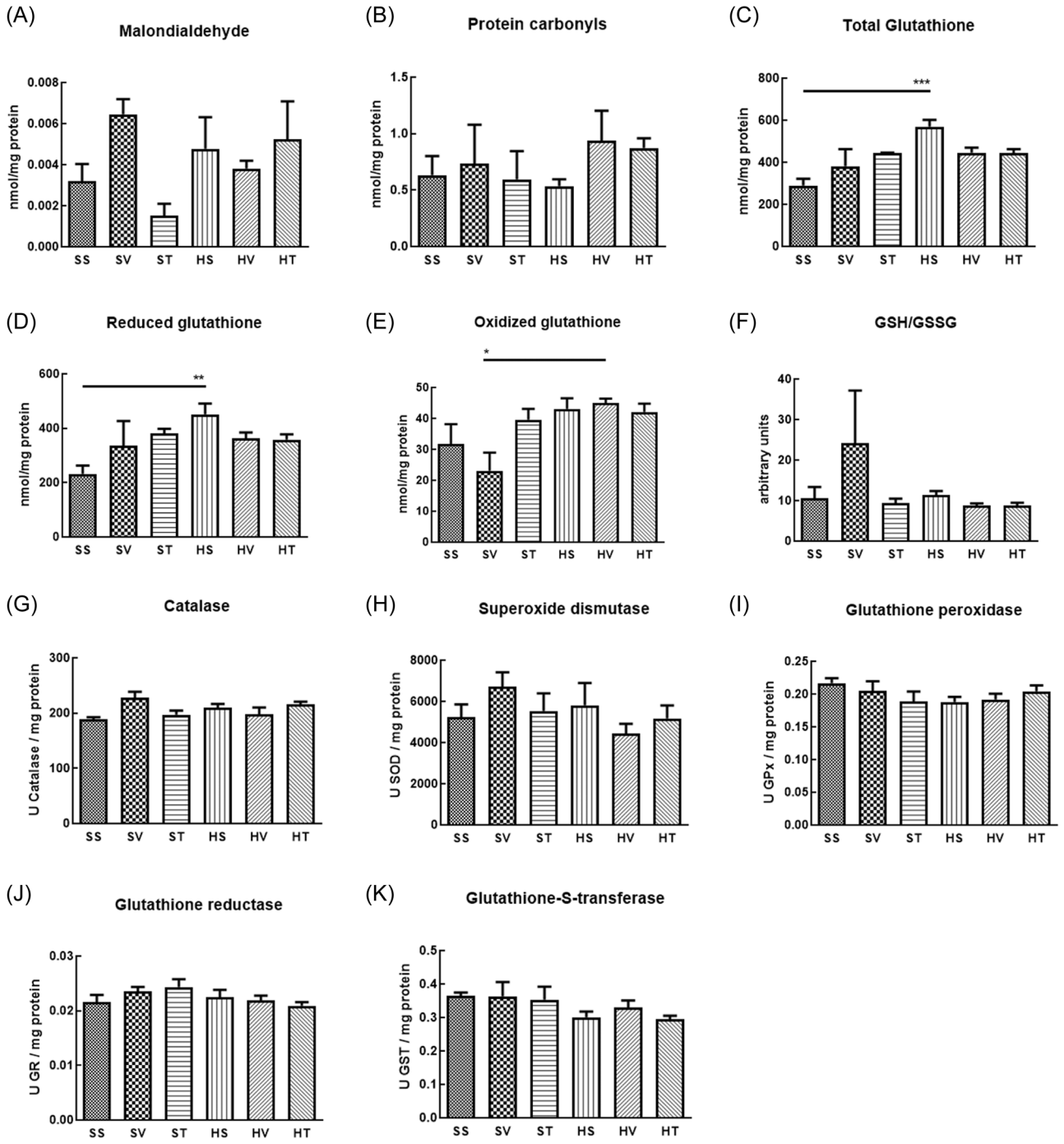

FIGURE 4 Effects of diet, voluntary physical activity, and endurance training on hepatic redox status parameters. (A) Malondialdehyde (nmol/mg protein); (B) protein carbonyl groups (nmol/mg protein); (C) total glutathione (nmol/mg protein); (D) reduced glutathione (GSH; nmol/mg protein); (E) oxidized glutathione (GSSG; nmol/mg protein); (F) ratio of GSH to GSSG (arbitrary units); (G) catalase (U catalase/mg protein); (H) superoxide dismutase (SOD; U SOD/mg protein); (I) glutathione peroxidase (GPx; U GPx/mg protein); (J) glutathione reductase (GR; U GR/mg protein); and (K) glutathione-S-transferase (GST; U GST/mg protein). Values are expressed as mean ± SEM. HS, sedentary animals with access to liquid high-fat diet; HT, endurance trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; SS, sedentary animals with access to standard control liquid diet; ST, endurance trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

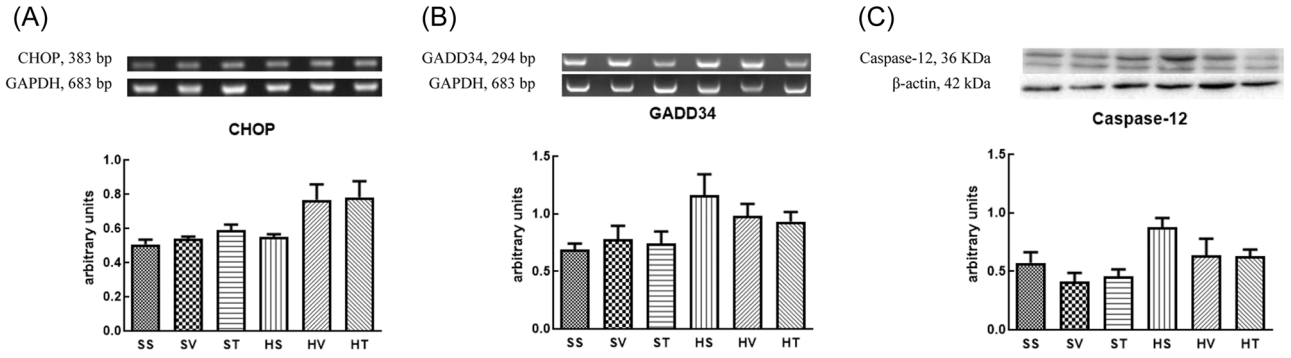

FIGURE 5 Effects of diet, voluntary physical activity, and endurance training on hepatic endoplasmic reticulum stress-related proapoptotic signaling parameters. Representative bands were obtained by semiquantitative Western blot analysis or reverse transcriptase-polymerase chain reaction. (A) C/EPB homologous protein (CHOP); (B) growth arrest and DNA damage-inducible protein (GADD34); and (C) cysteine-dependent aspartate specific protease (caspase)-12. Values are expressed as mean ± SEM. bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HS, sedentary animals with access to liquid high-fat diet; HT, endurance-trained animals with access to liquid high-fat diet; HV, voluntarily physically active animals with access to liquid high-fat diet; kDa, kilodalton; SS, sedentary animals with access to standard control liquid diet; ST, endurance-trained animals with access to standard control liquid diet; SV, voluntarily physically active animals with access to standard control liquid diet. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

insulin, proinflammatory, and glucocorticoid signaling pathways, which are activated by ER stress. In fact, markers of ER stress have been found in liver tissue samples from patients with NASH.[44]

Although there is already published evidence on the impact of PE on obesity-related ER stress,[41,42] the full extent of the molecular mechanisms involved is not yet totally unraveled nor is the impact of PE in their interconnection. To the best of our knowledge, we showed here, for the first time, that both preventive and therapeutic interventions using VPA and ET, respectively, positively modulated the unfolded protein response (UPR) in an animal model of HFD-induced NASH. The novelty of our study stands from the simultaneous evaluation of VPA and ET effects upon ER and oxidative stresses and proapoptotic signaling in an animal model of HFD-induced NASH.

Nowadays, ER is no longer solely considered as the calcium accumulating system and/or the protein synthesis machinery. In fact, ER has been suggested as one of the most important stress sensors of the cell.[22,23,25,26] Secreted and transmembrane proteins are folded in the ER; when the ER folding capacity is disturbed, UPR is triggered. UPR activation, being its first stage considered a prosurvival mechanism and a window of opportunity for restoration of cellular homeostasis,[45,46] depends on ER transmembrane proteins, namely PERK, IRE1α, and ATF-6, which dynamically adjust the protein folding capacity to the cell protein synthesis rate.[1,22,27,47]

In the present study, the results within the HFD arm displayed an apparent paradoxical pattern in which the expression of activated IRE1α (p-IRE1α) is significantly decreased by VPA while the expression of both uXBP1 and sXBP1 is significantly increased by ET.

The IRE1α–XBP1-mediated pathways are associated with the UPR proadaptive pathway, protecting the liver from ER stress-induced hepatic lipid accumulation and hepatic injuries.[46,48] Upon ER stress conditions, IRE1α splices uXBP1, via its RNase activity,[49,50] generating the functional sXBP1 that, in turn, activates the expression of a subset of UPR-associated regulators.[3,46,48] Additionally, IRE1α RNase activity also degrades several specific mRNAs and microRNAs (miRNAs), including itself, through a process called regulated IRE1-dependent decay (RIDD).[46,48,50–53] In HFD-induced obesity, chronic proinflammatory signaling causes IRE1α S-nitrosylation leading to a progressive decline in hepatic RIDD and IRE1α RNase-mediated splicing activity upon uXBP1. Oppositely, revealing a small cycle, inhibition of IRE1α *S*-nitrosylation enhances XBP1 splicing and reduces c-Jun *N*-terminal kinase (JNK) activity.[53] Deficiency in IRE1α RNase activity, caused by IRE1α S-nitrosylation, leads to an upregulation of a subset of miRNAs that are targeted by the IRE1α–RIDD pathway which downregulates genes that promote a NASH-like phenotype both in animal models and humans.[48] In the HFD arm of this study, VPA significantly decreased p-IRE1α and ET significantly increased both XBP1 forms; so VPA declined IRE1α activation while ET acted at the level of XBP1 production and splicing but only in an HDF context, as in the SCLD arm of the study no changes were observed for the three parameters. How do we reconcile all these observations to explain these puzzling, yet important, modulatory effects of PE upon UPR? We propose PE might modulate IRE1α activity-related

decline both in sXBP1 and RIDD, with VPA and ET behaving differently. Although the mechanism underlying modulation of IRE1α–XBP1 and IRE1α–RIDD pathways by VPA and ET can only be speculated at present, it does pose the interesting hypothesis that VPA and ET could differently decrease the IRE1α S-nitrosylation while increasing RIDD. However, future studies are needed to unlock the corresponding molecular mechanisms (as well as their PE specificity).

Additionally, our results showed an increased tendency for ATF-6 in HT when compared to HS and ST, which could suggest another adaptive prosurvival response induced by ET in HFD-fed animals. ATF-6 not only regulates genes involved in protein folding and ER-associated degradation but also activates uXBP1 transcription,[51,54] which mirrors the increased expression of uXBP1 (along with sXBP1) observed in HT when compared to HS and ST. Furthermore, after the release from BiP and its consequent activation, the PERK–eIF2α–ATF-4 pathway can also induce the expression of IRE1α.[54] However, while ATF-6 increases sXBP1 mRNA expression by increasing uXBP1 transcription, PERK–eIF2α–ATF-4 increases sXBP1 mRNA expression by increasing IRE1α expression.[54] This may explain the apparent paradoxical effects of PE in the HFD arm (decreasing pattern of eIF2α [by VPA and ET], p-eIF2α [by VPA], and p-IRE1α [by VPA] and increasing pattern of ATF-6, sXBP1, and uXBP1 [all three by ET]). In the SCLD, VPA-induced, only a negative impact upon total and activated eIF2α was observed.

Our data suggest that ET could function as an activator of sXBP1. As inhibition of sXBP1 activity appears to increase liver triglycerides content,[55] it is conceivable that the increased expression of sXBP1 induced by ET in HFD contributes to protecting the liver against lipid retention, which is in accordance with our previous study.[5]

Additionally, ATF-6 related modulation of peroxisome proliferator-activated receptor α and sterol regulatory element-binding protein 2 protects against hepatic steatosis by stimulating hepatic fatty acid oxidation and downregulating lipogenesis.[46,51,56,57] Together with the increased expression of sXBP1, which is an anti-lipogenic protein,[58] all these effects might have contributed to ET-induced NASH classification downgrading.

Reactive oxygen species production occurs as a by-product of the protein folding process[59,60] and glucocorticoids receptor knockdown decreases antioxidant protection (i.e., nuclear erythroid 2 p45-related factor [Nrf2]-dependent downregulation of GSH, SOD1 and SOD2, CAT, GPX, and GR).[61] PERK–eIF2α activation confers cellular antioxidant protection by inducing ATF-4 and Nrf2, key transcription factors in glutathione metabolism.[62] In this animal model, glucocorticoids receptor expression was significantly increased by ET in the HFD-arm of the protocol,[18] and VPA and ET modulated several UPR parameters, mostly in the HFD arm of the protocol. However, no significant changes were observed in oxidative damage biomarkers and antioxidant capacity-related elements (both Nrf2-independent and Nrf2-dependent) induced by PE. GSH depletion during HFD-induced UPR prompts increased oxidative stress and contributes to cell death.[28] So, the HFD-induced increase of GSx and GSH levels could a) represent an adaptive mechanism to cope with the expected oxidative stress imposed by HFD,[63,64] in a strain-specific dependent way,[65] and b) explain the lack of differences in the oxidative stress-induced cell damage among groups. Further studies are needed to clarify the intricacies of these complex results.

It is widely accepted that NASH-related cell death occurs under prolonged and/or unmitigated ER stress through several mechanisms, including (I) PERK–eIF2α–ATF-4–CHOP pathway; (II) IRE1α-induced activation of apoptotic signaling through apoptosis signaling-regulating kinase 1/JNK; (III) IRE1α-tumor necrosis factor receptor-associated factor 2 (TRAF2)-induced activation of caspase-12; and (IV) release of Ca^{2+} from the ER.[27] Although CHOP might be a key player in the modulation of ER stress-induced apoptosis,[66] caspase-12 is accepted as the specific molecule of ER stress-induced apoptosis.[67] GADD34 is downstream of the PERK pathway and is responsible for cell recovery from protein synthesis shut-off imposed by eIF2α inhibition,[68] once ER stress is resolved, to restore protein translation.[66] Nevertheless, sustained activation of the PERK–eIF2α pathway, and consequent overexpression of GADD34, results in an additional cargo to the already stressed ER milieu through the increment of newly synthesized protein, reinforcing the activation of cell-death pathways.[47]

Our HFD results suggest that eIF2α decrease (by VPA and ET) and p-eIF2α declining pattern (by VPA) might represent, on one hand, an adaptive ER stress response to avoid or reduce the increase of GADD34 expression, as this (if intense and/or prolonged) could facilitate a switch from the synthesis of prosurvival to proapoptotic proteins. On the other hand, it could also suggest that the ER stress is almost solved in these animals, and translational repression was not needed. In fact, no change was seen in CHOP or GADD34.

Additionally, the activation of and signaling by IRE1α plays an important role in determining cell fate.[49] IRE1α sustainably activated binds to TRAF2 leading to cell death, through JNK and ER membrane resident caspase-12 activation.[27,46,49,69] In line, as aforementioned, in the HFD arm, both VPA and ET significantly decreased JNK

activation[18] which almost matched the decrease of IRE1α activation. However, no change in caspase-12 expression was observed. Nevertheless, previous data showed that PE was able to reduce the activities of both caspase-8 and caspase-9.[70] Altogether, our results suggest that the antiapoptotic feature of PE lies in the reduction of both caspase-8 and caspase-9 activities[70] rather than in the abrogation of GADD34 and caspase-12 expression (disclaimed here).

NASH management requires a multidisciplinary and coordinated approach.[8,13] Although lifestyle modifications are the first-line option in NASH management, the understanding of the full extent of molecular mechanisms explaining PE effects on NASH-related hepatic ER stress, and its associated deleterious effects, including oxidative stress and ER stress-induced apoptosis, is far from complete; therefore, continued research is required.

Overall, our results seem to suggest that, at least in part, ET positively modulates this process.[5,18] Thus, exporting these results to humans helps to understand the mechanisms underlying the pathophysiology of the disease, but the translation should be done with caution. Unlike experimental animals, humans present gene variability that conditions this translation.[71–73]

5 | CONCLUSION

Overall, the combined variation of the three UPR arms suggests that HFD induced hepatic ER stress, while, at least partially, both VPA and ET positively modulated UPR by promoting cytoprotective survival response to ER stress. However, regarding the impact of VPA *versus*ET, and also according to our previous study,[18] it appears that exercise intensity, instead of volume, should be the essential feature of the exercise protocols, as it mediates a more determinant influence on exercise-induced cross-tolerance effects against the deleterious consequences of some pathological conditions. Taken together, our data support the idea that PE can be assumed as a potential protective nonpharmacological tool against NASH-related ER stress and its associated deleterious consequences through the dynamical modulation of the hepatic IRE1α–XBP1 pathway.

AUTHOR CONTRIBUTIONS

Emanuel Passos, António Ascensão, José Magalhães, and Maria J. Martins were responsible for study concept and design. Emanuel Passos and Inês O. Gonçalves took care of animals' treatment and exercise protocols. Emanuel Passos and Cidália Pereira performed all reverse transcriptase-polymerase chain reactions and Western blot analysis, and most liver biochemical assays. Ana Faria performed some biochemical analysis. Emanuel Passos drafted the manuscript. All other co-authors critically revised the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data described in this manuscript will be made available upon request pending application and approval or payment.

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CHAPTER 4: GENERAL DISCUSSION

GENERAL DISCUSSION

The prevalence of obesity-induced NASH dramatically increased in the last few years as a consequence of both sedentary lifestyle and excessive consumption of high-caloric food [6-13, 223, 224]. Although nowadays it remains incompletely understood, it is widely accepted that NASH pathogenesis is complex and multifactorial, implicating multiple parallel metabolic and molecular changes and converging signaling pathways [17, 33, 38, 225]. As detailed in the Introduction section, several insults act together or sequentially, sometimes on a background of genetic predisposition, to promote NAFLD and transition to NASH (and its progression) [17, 20, 22-30, 33, 38, 41-51, 140, 223-226]. The epidemic increase in the prevalence of NASH suggests that it may become the primary cause of liver transplantation in many countries in the coming years [29, 194]. Due to its global impact in terms of public health and health costs [197, 227], several medical associations initiated a "Call to Action" to issue guiding strategies for NASH management [198, 199]. Currently, numerous therapies are under investigation, pointing towards several pathological changes associated with NASH [199]. Even though the scientific community has been widely involved in a tremendous effort for developing novel therapies targeting NASH, approved pharmacological therapies are still lacking [29, 189], probably because of the incomplete knowledge regarding the mechanisms surrounding the disease. Taking into account that only a few of the investigated therapies have had their effectiveness confirmed in phase III trials, and some haven't had their benefits confirmed compared to placebo [189, 190, 194], the management of the gaps between clinical guidelines and clinical practice concerning NASH patients will continue. Until now, PE (being a non-pharmacological therapeutic strategy) seems to be the intervention that best addresses this highly unmet medical need [4, 55, 97, 203, 208, 216].

In fact, regular exercise training is widely accepted to have a strong and positive impact against several metabolic features associated to the development and progression of NASH in both humans and rodents [5, 55, 97, 204, 208, 209, 215, 228-231]. However, the set of mechanisms underlying how PE mitigates NASH is not yet completely known neither is unraveled the interconnection among these mechanisms.

Bearing this in mind, in the context of this work, liquid HFD was given to Sprague-Dawley male rats, as this model mimics certain aspects of human NASH [98]. Two different PE

models, life-long voluntary physical activity (VPA) and endurance training (ET), were applied in order to allow a better and deep "landscape" about the use of PE as a non-pharmacological therapeutic strategy against NASH and its consequent deleterious effects. Although the results of our study demonstrated that exercise training had a significant positive impact in anatomical and clinical features of NASH [**original research article (ORA) 1**], our novelty stands from the benefits obtained with PE upon the interestingly tightly interconnection between hepatic insulin, glucocorticoid and inflammatory signaling pathways (**ORA 2**) as well as ER and oxidative stresses and apoptotic signaling (**ORA 3**) in an animal model of HFD-induced NASH. Overall, we provide a better understanding of possible molecular mechanisms related to PE-induced protection against NASH and highlight UPR-related features behind exercise protection in the liver.

Although the "multiple hits" hypothesis is considered appropriate to explain NASH pathogenesis, hepatic fat accumulation is still an essential event. It is widely accepted that when the ability of adipose tissue to storage fat reaches its limit, the adipose tissue endocrine functions are altered causing the accumulation of ectopic fat in the liver [21, 27, 28, 30], which, unsurprisingly, may lead to hepatic lipotoxicity [110-112], a hallmark of NASH [21, 113, 117, 232]. As expected [98], **ORA 1** showed that sedentary HFD-fed animals presented the typical NASH hepatic tissue injury found in humans. Being PE accepted as an intervention that effectively mitigates this pathological condition [97, 209, 229], **ORA 1** showed that ET significantly counteracted the hepatic histological features induced by the HFD regimen (as confirmed by light electron microscopy) but not VPA. ET also significantly positively modulated visceral adiposity and all other obesity-related measures (with the exception of liver weight to body weight ratio) in both diet regimens. VPA did not significantly positively modulate the entire set of evaluated parameters in both branches; VPA induced a lower reduction of visceral adiposity (and visceral adiposity index) than ET and did not change liver weight in HFD branch, for example [55]. Therefore, ET might have reduced the release of FFA and pro-inflammatory molecules from the visceral depot directly to the liver, through the portal circulation, more than VPA [22, 45, 55, 105, 230].

ORA 2 showed that HFD-fed animals had significantly increased IL-1β (similarly for TGF-β and IL-6) in accordance with the histological findings observed in **ORA 1**. A key NASH

feature is lipotoxicity-induced hepatic inflammation, through JNK and NF-κB signaling pathways, and consequent increases of pro-inflammatory mediators, like IL-1β, IL-6, and TNF-α [21]. Although our results (**ORA 2**) showed an absence of activation of the IKK–NF-κB pathway and an absence of change of the p-JNK to JNK ratio in the HFD arm, both VPA and ET significantly reduced the expression of total JNK and the activation of JNK, suggesting thus that VPA and ET might have functioned similarly as JNK inhibitors. Additionally, both VPA and ET significantly reduced IL-1β and TGF-β in the HFD arm, while IL-6 and LIF were significantly reduced only by ET (a similar pattern being observed for VPA) (**ORA 2**). The absence of a significant reduction of both IL-6 and LIF by VPA in the HFD arm matches with the maintenance of liver histological degeneration in this animal group (**ORA 1)**. In fact, considering the HDF arm, the distinct strength of impact of VPA and ET in the above-mentioned parameters concurs with the histological data improvement (**ORA 1)**.

Our results from **ORAs 1 and 2** are corroborated by several other publications demonstrating that ET reduces the expression of pro-inflammatory molecules in the liver [55, 86, 208, 215, 233]. Even though previous studies demonstrated the positive effects of ET in the attenuation of liver fibrosis [215], to our knowledge, **ORA 2** demonstrated for the first time that both VPA and ET were able to significantly reduce the hepatic expression of TGF-β in an animal model of HFD-induced NASH, leading to a reduction in hepatic fibrosis and inflammation (as in line with others studies) [139, 140, 234]. Therefore, we propose that the observed reduction of the pro-inflammatory state induced by PE could be explained at least in part by the reduction of JNK signaling and TGF-β protein expression, in an ectopic hepatic fat accumulation dependent way.

Together, JNK and NF-κB inflammatory signaling coupled with hepatic lipotoxicity is accepted to induce the hepatic insulin resistance observed in NASH [21], in a vicious circle manner, i.e., lipotoxicity promotes inflammation and insulin resistance, then insulin resistance aggravates lipotoxicity [21]. Although we did not observe a decrease in plasma glucose concentration induced by PE, neither in SD nor in HFD arms (**ORA 1**), we found that ET significantly induced insulin sensitivity improvement in the animals fed with both types of diets (**ORA 1**), as previously reported by others [208]. It is widely accepted that NASH-related impaired insulin signaling is characterized by increased inhibitory IRS-1^{Ser307} phosphorylation, being JNK pathway directly implicated in this

process [129, 135, 235-238]. In accordance, as depicted in **ORA 2**, both VPA and ET significantly counteracted insulin signaling inhibition imposed by IRS-1^{Ser307} phosphorylation HFD-induced. Taken together, data from **ORAs 1 and 2** strongly suggest that PE could be an excellent non-pharmacological therapeutic strategy to break the "bridge" between hepatic lipotoxicity, inflammation and insulin resistance NASH-related. Additionally, in both types of diets, ET significantly decreased AKT activation (a similar pattern was observed for VPA), while both VPA and ET significantly decreased AKT in the HFD arm [a similar pattern was observed in the standard diet (SD) arm], and ET significantly decreased the p-AKT/AKT ratio in HFD-fed animals (a similar pattern being observed for VPA) (**ORA 2**). Taking into consideration that in insulin resistance animal models, increased basal and/or insulin-stimulated AKT activation occurs in the liver (along with a worse metabolic profile, mitochondrial dysfunction, and increased oxidative stress [239-241]), we accordingly found out not only a consistent modulation towards reduction of the *AKT system* but also an increase of the insulin sensitivity index by ET (but not by VPA) in the HFD arm (**ORA 2**). These findings agree with the reduction of the inhibitory phosphorylated IRS-1 to total IRS-1 ratio and the overall changes induced in the JNK signaling as well as in the pro-inflammatory profile induced by ET and VPA. In fact, pro-inflammatory cytokines activate extracellular signal-regulated kinase (ERK) [131, 242] what in turn desensitizes the insulin signaling through IRS-1/2 inhibitory phosphorylation [131, 243]. In HFD rats, both VPA and ET modulated ERK signaling most probably aiming to protect the insulin signaling - in complete accordance with the reduction observed in the ratios of p-IRS-1/total IRS-1 and p-AKT/total AKT, JNK signaling and pro-inflammatory molecules (always significantly for ET).

Additionally, the increase of glucocorticoid metabolism, through 11β-HSD1, along with the increase of PTP1B, plays a pivotal role in the onset and/or progression of the hepatic insulin resistance. Furthermore, long-term and persistent elevated glucocorticoid levels are critical in the pathogenesis and/or progression of NAFLD/NASH [55, 105, 244-246]. In fact, the hepatic activity and/or mRNA expression of 11β-HSD1 is strongly regulated by the disease progression and worsening of inflammation and liver injury: the activity of 11β-HSD1 decreases in simple steatosis but increases in NASH [55, 226]. For this reason, 11β-HSD1 has been suggested as a new target in pharmacotherapy of NASH [246, 247]. Even though our results showed an absence of change induced by diet

regimen *per se* in 11β-HSD1 protein content and PTP1B mRNA expression, in **ORA 2**, we showed for the first time that both VPA and ET may function in a preventive and therapeutic way as insulin sensitizers analogs, respectively, by significantly reducing the hepatic expression of 11β-HDS1 in both diets regimen (a tendency was observed for ET in the HFD arm) while ET also reduced the expression of PTP1B in the HFD-animals.

Serine^{307} phosphorylation of IRS-1 increases with glucocorticoids and, consequently, the affinity of IRS-1 for the insulin receptor decreases and the degradation of IRS-1 increases [248, 249]. So, the inhibition and/or the deletion of 11β-HDS1 could decrease insulin resistance [168, 248-251]. This is in agreement and reinforces our previous idea that PE could represent a non-pharmacological alternative way to the prescription of insulin sensitizer analogs. Additionally, since PTP1B inhibits the insulin signaling cascade by dephosphorylating (and switching off) the tyrosine residues of insulin receptor and IRS-1 [252-256], the decreased activity of this phosphatase could mean that more adequate phosphorylation levels of insulin receptor and IRSs would be maintained assuring signal propagation in the insulin signaling [136, 138, 252, 257]. In accordance, as depicted in **ORA 2**, ET reduced the PTP1B in the HFD arm, which is in accordance with improved ISI as well as with the overall decreased pro-inflammatory signaling (as shown by p-JNK, JNK, IL-1β, IL-6 and LIF lessening). Considering that the expression of PTP1B is induced by pro-inflammatory signaling [126], data from **ORA 2** strongly suggest that PE could be an excellent non-pharmacological analog of PTP1B inhibitors to break the thigh interconnection between hepatic pro-inflammatory signaling, PTP1B and insulin resistance NASH-related.

In addition, the fact that both PTP1B and 11β-HSD1 occur on the cytosolic surface of the ER [138], and the silencing of PTP1B in hepatic cell lines or mouse liver protects against pharmacologically and/or HFD-induced ER stress [258], prompted us to hypothesize a beneficial effect of PE on hepatic ER stress NASH-related.

The “multiple hits” hypothesis for the onset of NAFLD and progression of NASH takes into account the involvement of prolonged ER stress in the process [4, 5, 9, 17, 22, 41, 42, 44, 51, 54, 55, 59, 60, 67-70]. In fact, in human NASH liver samples, markers of prolonged ER stress have been found [134]. Nevertheless, the mechanisms of NASH-related ER stress are complex, not completely understood and most of them are nonexclusive [67, 134, 143, 147-156], with ER stress-associated hepatic lipotoxicity, pro-

inflammatory signaling, insulin resistance, increased oxidative stress, mitochondrial dysfunction and Ca^{2+} homeostasis disruption as well as increased/dysregulated apoptotic signaling involved in this process, in a vicious cycle manner [9, 67, 157-161]. Results from **ORA 3** did not show activation of the three UPR branches (PERK, IRE1α and ATF-6) or increase of BiP expression induced by HFD *per se*. Nevertheless, when we analyzed the role of both VPA and ET upon hepatic ER stress markers, our results displayed, at the first glance, within the HFD arm, a contradictory pattern of UPR in which activated IRE1α was significantly decreased by VPA while both unspliced X-box binding protein 1 (uXBP1) and spliced XBP1 X-box binding protein 1 (sXBP1) were significantly increased by ET. Bearing in mind this complex puzzle, we speculated VPA and ET modulated the IREα/XBP1 pathway differently.

Since sXBP1 inhibition seems to increase liver TGs content [259], we can speculate that ET-induced increased sXBP1 expression, within the HFD arm, protects HET animals from hepatic steatosis and matches with the improvement of liver histological data observed in HET animals (**ORA 1)**. Additionally, we also observed an increase in uXBP1 induced by ET in the HFD arm. Although uXBP1 has an extremely short half-life [155, 260], the efficiency of XBP1 mRNA splicing by active IRE1α is controlled by uXBP1 [155, 261, 262], which is in accordance with and could explain why the increased expression of uXBP1 parallels the expression of sXBP1 in the ET group.

A significant decrease of p-IRE1α induced by VPA was observed within the HDF arm. p-IRE1α observed in VPA animals in the HFD branch (**ORA 3**) revealed a quite similar pattern to 11β-HSD1 and p-JNK (**ORA 2**) in the same group of animals. Considering that increased activation of the IRE1α pathway under sustained ER stress mediates increased expression of pro-inflammatory signaling, such as IL-1β, through the activation of JNK [5, 110, 133], and since the level of 11β-HSD1 activity could be upregulated by pro-inflammatory cytokines, such as IL-1β, we could speculate that the reduction of 11β-HSD1 induced by VPA in HFD observed in ORA 2 could be explained at least in part by the reduction of IL-1β, probably by reducing the level of p-JNK.

Additionally, we observed that in the HFD arm both VPA and ET significantly decreased eIF2α and VPA led to a declining pattern upon p-eIF2α. An intense and/or prolonged increase of GADD34 could promote a switch from prosurvival to proapoptotic ER response. Then, the abovementioned results may a) represent an attempt to prevent or

reduce GADD34 increase (preventing or reducing proapoptotic protein synthesis), or b) suggest that the translational repression was not necessary since ER stress was practically solved in these animals. Taking together, these results could explain at least in part the absence of significant impact observed in both in CHOP and GADD34.

Several studies have shown indisputable evidence that increased ectopic hepatic fat accumulation, characteristic of NASH, increases hepatic ROS production most probably due to an increased electron overflow in the mitochondrial electron transfer chain as a consequence of increased β-oxidation [21, 107, 263, 264]. In addition, increased oxidative stress disturbs the complex redox homeostasis existent in the lumen of ER [107]. Furthermore, the production of ROS is a by-product during the protein folding process, and may have a detrimental effect on the structure and function of both mitochondria and ER in a vicious cycle manner [169, 265]. Although VPA and ET modulated several features of NASH, such as hepatic inflammation and insulin signaling, as well as glucocorticoids signaling and several parameters of UPR, no significant changes were induced by PE in oxidative damage and antioxidant capacity markers. Nevertheless, we observed an unexpected HFD-induced increased GSX and GSH levels. We suspected that this result could represent an adaptive mechanism to counteract the exacerbated oxidative stress created by HFD. So, the effects of PE training on NASH-induced changes in oxidative stress markers and the cell capacity to buffer ROS through both enzymatic and not enzymatic antioxidant remain to be elucidated in the future studies.

Under intense and/or chronic ER stress conditions, UPR behaves like a binary switch between the attempt to restore ER homeostasis and cell death [9, 155, 266-270], which suggest that the kinetics and amplitude of UPR signaling are tightly regulated at different levels [155], being the first phase of UPR a window of opportunity to restore homeostasis. Although PE regimens did not produce a significant effect on CHOP, GADD34 and caspase-12, the effects of both VPA and ET decreased eIF2α in HFD branch as well as the tendency of VPA on p-eIF2α (**ORA 3**) coupled with the decreasing activation of JNK by PE in the HFD arm observed in **ORA 2** could suggest that both VPA and ET could be able to revert the possible activation of NASH-related ER stress-induced apoptosis, through IRE1α-JNK pathway by decreasing the activation of JNK (**ORA 2 and 3**).

Although the overall results regarding PE effects upon the hepatic ER stress, and its consequent deleterious effects are promising, data relating PE training to HFD-induced hepatic ER stress are limited, and wide-ranging cellular and molecular mechanism remain elusive due to the lack of detailed experimental research. In order to further increase our knowledge regarding the molecular mechanisms that underlie the multiple positive effects of PE, either as a preventive or as a therapeutic tool to counteract NASH development and progression, more studies are needed. Different PE approaches and/or combinations should be tested.

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CHAPTER 5: GENERAL CONCLUSIONS

General conclusions

Based on the results that emerged from our **ORAs 1, 2 and 3**, the following conclusions may be highlighted:

a) ET could be a non-pharmacological therapeutic strategy to counteract both the development of hepatic lipid droplet accumulation and inflammation, since it reverted the anatomical and clinical/histological alterations observed in an animal model of HFD-induced NASH.

b) PE positively modulated a) JNK signaling as well as IL-1β, IL-6, LIF and TGF-β and b) insulin signaling, most probably through JNK signaling, PTP1B and 11β-HSD1, in an animal model of HFD-induced NASH.

c) PE was capable to positively and dynamically modulate the NASH-related UPR by enhancing pro-survival response, while down-regulating pro-death, through the IREα/XBP1 signaling pathway, in an animal model of HFD-induced NASH.

In general, these results allow us to suggest that physical exercise can fill the existing gap in clinical management of NASH and be recommended as a non-pharmacological strategy in the prevention and/or treatment of NASH.

CHAPTER 6: GENERAL REFERENCES

GENERAL REFERENCES

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