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Evaluation of cannabidiol influence on lipid metabolism in the skeletal muscle in a rat model of obesity induced by a high-fat diet

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List of publications

Article type	Number	Impact Factor	MNiSW points
Articles included in the dissertation	3	12.262	340
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Summary	15	29.511	780

Doctoral dissertation

Evaluation of cannabidiol influence on lipid metabolism in the skeletal muscle in a rat model of obesity induced by a high-fat diet

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1. Articles included in the dissertation

Name of the journal	Title of the article	Impact Factor	MNiSW	Date of publication	Type of publication
Frontiers in Endocrinology	*Phytocannabinoids: useful drugs for the treatment of obesity? Special focus on cannabidiol*	3.634	100	4.03.2020	Article No. 1 – review article
Biomolecules	*Chronic cannabidiol administration attenuates skeletal muscle de novo ceramide synthesis pathway and related metabolic effects in a rat model of high-fat diet-induced obesity*	4.082	100	26.08.2020	Article No. 2 – original article
Nutrients	*Attenuation of oxidative stress and inflammatory response by chronic cannabidiol administration is associated with improved n-6/n-3 PUFA ratio in the white and red skeletal muscle in a rat model of high-fat diet-induced obesity*	4.546	140	11.05.2021	Article No. 3 – original article

2. List of abbreviations

2-AG - 2-arachidonoylglycerol

4-HNE - 4-hydroxynonenal

5-HT1A - serotonin receptor 1

5-LO - 5-lipoxygenase

15-LO - 15-lipoxygenase

β-HAD - β-hydroxyacyl-CoA dehydrogenase

Δ9-THC - Δ9-tetrahydrokannabinol

ACS - acyl-CoA synthetase

AEA - anandamide

AGE - advanced glycation end products

ALP - alkaline phosphatase

ASAH1 - acid ceramidase

AS160 - Akt substrate of 160 kDa

ATP - adenosine triphosphate

BCA - bicinchoninic acid method

Bcl-2 - B cell lymphoma 2

BSA - bovine serum albumin

CAT - catalase

CB_1 - cannabinoid receptor 1

CB_2 - cannabinoid receptor 2

CBD - cannabidiol

CER - ceramide

CerS - ceramide synthase

CoA - coenzyme A

COX-1 - cyclooxygenase-1

COX-2 - cyclooxygenase-2

CO_2 - carbon dioxide

CPT I - carnitine palmitoyltransferase I

DAG - diacylglycerols

DGAT - diacylglycerol acyltransferase

ECS - endocannabinoid system

eCBome - endocannabinoidome

FAs - fatty acids

FAAH - fatty acid amide hydrolase

FABPc - cytosolic fatty acid binding protein

FABPpm - plasma membrane fatty acid binding protein

FAME - fatty acid methyl esters

FAT/CD36 - fatty acid translocase

FATP-1 - fatty acid transport protein 1

FATP-4 - fatty acid transport protein 4

FFA - free fatty acids

GLC - gas-liquid chromatography

GLUT1 - glucose transporter 1

GLUT4 - glucose transporter 4

GPR18 - G protein-coupled receptor 18

GPR55 - G protein-coupled receptor 55

GSK-3 - glycogen synthase kinase 3

HPLC - high-performance liquid chromatography

HRP - horseradish peroxidase

IL-6 - interleukin 6

IRS-1 - insulin receptor substrate 1

IR - insulin resistance

LASS5 - ceramide synthase 5

LCFA - long-chain fatty acids

MAGL - monoacylglycerol lipase

MDA - malondialdehyde

MMP-2 - matrix metalloproteinase-2

MMP-9 - matrix metalloproteinase-9

NF-κB - nuclear factor- κB

Nrf2 - nuclear factor erythroid 2-related factor 2

PAP - phosphatide phosphatase

PDH - pyruvate dehydrogenase

PDK - 3-phosphoinositide dependent protein kinase-1

PIP_3 - phosphatydilinositol (3,4,5)-triphosphate

PI3K - phosphatidylinositol 3-kinase

PKB/Akt - protein kinase B

PKC - protein kinase C

PL - phospholipids

PPARα - peroxisome proliferator-activated receptor α

PPARγ - peroxisome proliferator-activated receptor γ

PP2A - phosphatidyl phosphatase 2A

PVDF - polyvinylidene fluoride

SFA - sphinganine

SFA1P - sphinganine-1-phosphate

SFO - sphingosine

SOD2 - superoxide dismutase 2

SPHK2 - sphingosine kinase 2

SPTLC1 - serine palmitoyltransferase, long chain base subunit 1

S1P - sphingosine-1-phosphate

TAC - total antioxidant capacity

TAG - triacylglycerols

TCA - tricarboxylic acid

TLC - thin-layer chromatography

TNF-α - tumor necrosis factor α

TRPV1 - transient receptor potential vanilloid type 1

T2DM - type 2 diabetes mellitus

3. Introduction

3.1 Energy substrates metabolism in the skeletal muscle

Skeletal muscles play a pivotal role in the utilization of energy substrates, such as glucose and fatty acids (FAs), due to their high metabolic activity and large mass (approximately 40% of body mass) [1]. Muscle tissue accounts for 80% of the insulin-dependent postprandial glucose uptake, therefore, following a meal, glucose serves as the main source of fuel for energy production in skeletal muscle. However, during the fasting state, muscle glucose uptake is low, and plasma FAs level is elevated, thus the principal energy source is fat oxidation. [2]. The ability of muscle tissue to switch from glucose oxidation during the postprandial conditions to fatty acid oxidation during the state of starvation is called metabolic flexibility. Moreover, the utilization of energy substrates varies significantly depending on the composition of the skeletal muscle fibers (oxidative vs. glycolytic) due to the different content of mitochondria and their capacity to oxidize fat, resulting in a variable effect of FAs on glucose utilization in red and white skeletal muscles [3].

The postprandial increase in plasma glucose concentration stimulates the secretion of insulin, which through activating the cell signaling cascade can initiate skeletal muscle glucose uptake. Glucose transport in skeletal muscle occurs mainly via facilitated diffusion involving the glucose transporters 1 and 4 (GLUT1 and GLUT4). GLUT1 is restricted to the cell surface, whereas GLUT4, which is predominantly expressed in skeletal muscles, is localized intracellularly from where it is are rapidly translocated to the plasma membrane in response to exercise, hypoxia, and insulin-triggered series of signaling events [4]. Insulin by binding to alpha subunits of insulin receptor results in the autophosphorylation of the intracellular beta subunits, which increases the activity of kinases and phosphorylates docking protein, insulin receptor substrate 1 (IRS-1) [5]. Subsequently, IRS-1 activates its downstream molecular target phosphatidylinositol 3-kinase (PI3K), which after association with the plasma membrane, phosphorylates inositol phospholipids and generates phosphatydilinositol (3,4,5)-triphosphate (PIP_3). The next stage of insulin signal transduction is the junction of PIP_3 with 3-phosphoinositide dependent protein kinase-1 (PDK1). PDK1 phosphorylates both protein kinases B (PKB/Akt) and protein kinases C (PKC) [6]. PKB/Akt activation leads to the phosphorylation of the AS160 protein, one of the PKB/Akt substrates involved in the regulation of GLUT4 translocation [7]. The entire cascade of insulin signaling results in the translocation of vesicles containing GLUT4 from intracellular stores to the cell surface, allowing glucose to enter the cell [8].

Long-chain fatty acids (LCFA) are transported across the plasma membrane of skeletal muscle via simple diffusion along their concentration gradient or may be facilitated by membrane-associated proteins [9]. To date, among the known transporters of fatty acids are fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), and a family of fatty acid transport proteins (FATP1,4), which are coexpressed in the skeletal muscle [10]. The presence of CD36 not only on the cell surface but also in intracellular storage depots, especially in endosomes, has suggested that translocation of CD36 from endosomes to the plasma membrane may enhance FAs uptake. The aforementioned mechanism is analogous to the well-known regulation of glucose uptake which relies on the translocation of the GLUT4 from the intracellular storage to the cell membrane [9]. Moreover, recent studies have shown that insulin, through activation of the signaling pathway related to the PI3K cascade, causes translocation of CD36 from intracellular compartments to the surface of the cell membrane, which determines the increase in intracellular LCFA transport [11]. In addition, the expression of CD36 has been demonstrated in the mitochondrial membranes, however, its role is not yet fully understood; probably this protein interacts with carnitine palmitoyltransferase I (CPT-I) in the regulation of LCFA transport into the mitochondrial matrix and affects the rate of LCFA oxidation (β-oxidation process) in skeletal muscle [12]. On the inner side of the plasma membrane, integral sarcolemmal fatty acid transport proteins can provide a docking site for the cytosolic fatty acid binding protein (FABPc) or fatty acid-acting enzymes (such as acyl-CoA synthetase - ACS), making the fatty acids readily available for subsequent transport and/or enzymatic conversion [13]. Upon entering the cells, LCFA forms complexes with coenzyme A (CoA) generating LCFA-CoA, which are channeled toward β-oxidation in the mitochondria or to lipid storage depending on metabolic requirements.

During starvation or exercise, LCFA-CoA are transferred to the mitochondria where they cross the mitochondrial membrane with the participation of CPT I and possibly FAT/CD36 transporter. Within the mitochondrial matrix, LCFA undergoes numerous regulated processes in which they are oxidized to carbon dioxide (CO_2) [14]. In particular, LCFA are degraded in the β-oxidation process, where the enzyme β-hydroxyacyl-CoA dehydrogenase (β-HAD) plays a key role and is involved in the generation of acetyl-CoA, which enters the tricarboxylic acid cycle (TCA), enabling adenosine triphosphate (ATP) synthesis through oxidative phosphorylation [14].

Under postprandial conditions, an increase in plasma glucose levels stimulates insulin secretion and the resulting hyperinsulinemia inhibits lipolysis, leading to a reduction in the

concentration of FAs in the plasma, followed by a decrease in the rate of lipid oxidation. Subsequently, intracellular LCFA undergoes the esterification process and they are channeled towards lipid storage in myocytes primarily as triacylglycerols (TAG) in lipid droplets [15]. TAG is considered to be a relatively safe lipid fraction with no intracellular lipotoxic effects, as some studies have shown that both the inhibition and activation of intramuscular TAG hydrolysis do not significantly affect the mitochondrial function and insulin sensitivity of cells [16]. However, in the course of high-fat feeding, excessive intracellular transport of LCFA, exceeding the oxidative capacity of the mitochondria, results in intramyocellular lipid accumulation, not only in the TAG fraction but also in the fractions of diacylglycerols (DAGs), as well as ceramides (CER) belonging to the sphingolipids family [17]. DAG is an intermediate formed in the TAG synthesis process, resulting from LCFA-CoA esterification to glycerol-3-phosphate and the subsequent dephosphorylation reaction of phosphatidic acid with the participation of the enzyme phosphatide phosphatase (PAP). The reaction directly leading to the formation of TAG is the acylation of DAG under the influence of diacylglycerol acyltransferase (DGAT) [18]. The DAG acts as an important second messenger involved in cell signaling, although it is considered as a molecule responsible also for insulin resistance (IR). It disrupts the downstream insulin signaling pathway by activating both classical and atypical PKC, which dephosphorylate IRS1 and thus block further insulin signaling [15]. Ceramide is another lipid metabolite that is linked with increased muscle lipid accumulation and IR. It is produced via de novo synthesis from palmitoyl-CoA or it can be generated from sphingomyelin, a phospholipid component of plasma membranes [19]. De novo CER synthesis takes place within the endoplasmic reticulum and begins with a serine and palmitoyl-CoA condensation reaction catalyzed by serine palmitoyltransferase (SPT) to produce 3-ketosphinganine. Subsequently, the reactions lead to the production of sphinganine (SFA), which after acetylation by ceramide synthase enzymes (CerS1-6) forms dihydroceramide and further CER with the participation of dehydroceramide desaturase [20]. Emerging evidence points out that CER interferes with glucose uptake and impairs the storage of nutrients such as TAG and glycogen as a result of attenuating insulin signal transmission through activation of atypical PKC isoforms (PKCζ/λ) and phosphatidyl phosphatase 2A (PP2A), which inhibits the phosphorylation of PKB/Akt. In both cases, inactivation of PKB/Akt results in inhibition of intramuscular glycogen synthesis as well as blocking the translocation of GLUT-4 to the plasma membrane, causing a decrease in insulin-dependent intracellular glucose transport [20].

Mounting evidence suggests that the accumulation of intramuscular lipids, in particular: diacylglycerols and ceramides, is related to the emergence of insulin resistance, the occurrence of which strongly correlates with the pathogenesis of numerous chronic diseases such as metabolic syndrome and type 2 diabetes mellitus (T2DM). This notice is essentially important as intramuscular accumulation of bioactive lipids occurs in obesity, suggesting a relationship between intramuscular lipid content and IR. These findings imply that lipid accumulation in the skeletal muscle is a key contributor to IR, although the exact mechanism remains to be clarified.

3.2 The endocannabinoid system in the skeletal muscle

The endocannabinoid system (ECS) plays a pivotal role in the regulation of the body's energy homeostasis and is part of a larger family of signaling lipids termed the endocannabinoidome (eCBome). The eCBome encompassing endogenous ligands called endocannabinoids (e.g., N-arachidonoylethanolamine - anandamide (AEA) and 2-arachidonoylglycerol (2-AG)) and their numerous long-chain fatty acid-derived congeners, the ligand metabolic enzymes (e.g., fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL)), as well as the receptors that respond to the components of *Cannabis* plant [21,22]. In addition, to the "classic" cannabinoid receptors (CB_1 and CB_2), many other molecular targets have been identified in common with ECS, including thermosensitive transient receptor potential (TRP) channels (such as vanilloid receptor type-1 - TRPV1), orphan G protein-coupled receptors (such as GPR55 and GPR18), and peroxisome proliferator-activated receptors (such as PPARα and PPARγ) [23]. In recent years, it has been shown that the ECS is overactivated in the course of obesity, which increases food intake and promotes energy storage [24]. As a result, higher ECS tone contributes to body fat accumulation and has been linked to the development of obesity-related metabolic abnormalities [25,26]. This is supported by several studies reporting a strong association between high plasma endocannabinoids levels, and IR in a variety of tissues, including skeletal muscle [27,28]. In addition, CB_1 activation has been shown to decline mitochondrial biogenesis in muscle tissue, reducing their level and negatively affecting oxidative phosphorylation [29]. In contrast, recent studies have shown that decreased ECS activity in skeletal muscle results in increased insulin sensitivity and glucose uptake [30,31].

3.3 The role of cannabidiol in the regulation of lipid metabolism in the skeletal muscle

The *Cannabis sativa* plant has been used for recreational and medical purposes for hundreds of years [32]. Cannabidiol (CBD) right after Δ9-tetrahydrocannabinol (Δ9-THC) is the most abundant phytocannabinoid in the *Cannabis* plant [33]. However, CBD has recently emerged as a therapeutic agent for many pathological conditions since it is devoid of psychoactive side effects and has an excellent safety profile unlike Δ9-THC [34]. In addition, many of its beneficial properties have been demonstrated, including anti-inflammatory, anti-oxidant, anti-convulsant, anti-cancer, and neuroprotective, suggesting a therapeutic application in numerous diseases, such as epilepsy and ischemia [35–37]. Recently, research has also indicated the potential therapeutic effect of CBD in the treatment of obesity and its metabolic complications [38–40]. CBD produces its effects through many different mechanisms, which illustrates its complex pharmacology, that is not yet fully understood. In the case of the cannabinoid receptors CB_1 and CB_2, CBD has been found to have a very low affinity for them, while it affects many other molecular targets to a much greater extent [41]. In particular, CBD has been shown to act as an agonist at the TRPV1, serotonin receptor 1 (5-HT1A), as well as PPARα and PPARγ, while on the other hand, it substantially antagonizes the GPR55 receptor [42,43]. Furthermore, both animal and human studies reported that CBD is able to modulate the ECS activity, mainly by inhibiting AEA uptake and preventing its hydrolysis by FAAH [33]. Modulating the tone of this system has great therapeutic promise for a wide range of diseases, ranging from mental health disorders, pain, and metabolic diseases with respect to the treatment of obesity and related disturbances. Overall, the unique pharmacological properties of CBD make this compound very attractive for therapeutic applications.

Detailed information on the endocannabinoid system and cannabidiol can be found in the following manuscript included in the dissertation:

a. Article No. 1 - Bielawiec P., Harasim-Symbor E., Chabowski A.: *Phytocannabinoids: useful drugs for the treatment of obesity? Special focus on cannabidiol*. Frontiers in Endocrinology, 2020, 11, 1–11.

4. Aims

Currently, obesity is one of the most common serious medical concerns worldwide. Its increasing prevalence has been attributed to a number of factors, including excessive food consumption, sedentary lifestyle, as well as environmental and genetic factors. Obesity along with IR are risk factors predisposing to T2DM, metabolic syndrome or cardiovascular diseases. In the course of obesity, increased availability of FAs in the diet leads to excessive storage of lipids in adipocytes and, subsequently, in other metabolically active tissues such as liver, cardiac and skeletal muscle. Recent reports indicate that one of the key systems involved in the development of obesity is the ECS. It is involved in the regulation of numerous physiological processes, including appetite, cellular metabolism, and energy homeostasis. It has also been shown that the ECS is the site of interaction of phytocannabinoids, which are compounds of plant origin (*Cannabis sativa*). Among phytocannabinoids isolated from the *Cannabis sativa* plant, CBD is characterized by a great safety profile and lack of psychoactive properties. Research in recent years has contributed to understanding CBD's numerous therapeutic effects, including its anti-inflammatory and antioxidant effects. It is also considered that CBD may have beneficial effects in the treatment of obesity, although its exact mechanisms of action remain unknown.

Taking the above into account, the aim of the conducted research was:

1. Assessment of cannabidiol influence on the plasma concentration of glucose and insulin, the total expression of glucose transporters, as well as proteins and their phosphorylated forms involved in the insulin signaling pathway in skeletal muscle in a rat model of high-fat diet-induced obesity,
2. Assessment of cannabidiol influence on the content of selected lipid fractions (FFA, DAG, TAG, PL) and sphingolipids (including CER) as well as the total expression of selected fatty acid transporters in skeletal muscle in a rat model of high-fat diet-induced obesity,
3. Assessment of cannabidiol influence on the composition of individual lipid fractions (FFA, DAG, TAG, PL) in skeletal muscle in a rat model of high-fat diet-induced obesity.

5. Materials and methods

Detailed information on the applied experimental model and research methodology can be found in the following manuscripts included in the dissertation:

a. Article No. 2 - Bielawiec P., Harasim-Symbor E., Konstantynowicz-Nowicka K., Sztolsztener K., Chabowski A.: *Chronic cannabidiol administration attenuates skeletal muscle de novo ceramide synthesis pathway and related metabolic effects in a rat model of high-fat diet-induced obesity*. Biomolecules, 2020, 10(9),1241, 1-16.
b. Article No. 3 - Bielawiec P., Harasim-Symbor E., Sztolsztener K., Konstantynowicz-Nowicka K., Chabowski A.: *Attenuation of oxidative stress and inflammatory response by chronic cannabidiol administration is associated with improved n-6/n-3 PUFA ratio in the white and red skeletal muscle in a rat model of high-fat diet-induced obesity.* Nutrients, 2021, 13(5), 1604.

5.1 Animals and experimental protocol

The experiment was conducted on male Wistar rats (weighing approximately 70-100 g) purchased from the Center for Experimental Medicine of the Medical University of Bialystok, Poland. The animals were housed in standard plastic cages under controlled animal holding conditions (22 ± 2 °C with a reversed light-dark cycle of 12 h/12 h) with unlimited access to drinking water and commercial laboratory chow (Labofeed B, Animal Feed Manufacturer "Morawski", Kcynia, Poland). The protocol of the study was evaluated and approved by the Animal Ethics Committee in Olsztyn (No. 71/2018). The animals, after one week period of acclimatization, were randomly assigned to four experimental groups: (1) control group - rats fed a standard rodent diet (kcal distribution: 12.4% of energy from fat, 57.1% from carbohydrates, and 30.5% protein), (2) CBD group - rats fed a standard diet and treated with CBD, (3) HFD group - rats fed a high-fat diet (kcal distribution: 60% of energy from fat, 20% from carbohydrates, and 20% protein), and (4) HFD + CBD group - rats fed a high-fat diet and treated with CBD. Each experimental group included 10 rats and the total time course of feeding rats with the standard laboratory chow or a high-fat diet lasted seven weeks. The animals, starting at the beginning of the sixth week, received injections of CBD or its vehicle for the consecutive 14 days of the experiment. Respective control and HFD-fed groups received intraperitoneal (i.p.) injections (once a day) of

synthetic CBD (purity: ≥99%; THC Pharm GmbH, Frankfurt, Germany) in a dose of 10 mg/kg of body mass or its solvent (3:1:16, ethanol, Tween-80, and 0.9% NaCl). Throughout the whole experiment, body mass was monitored. 24 hours after the last dose of CBD or its solvent, rats were anesthetized by i.p. injections of pentobarbital (80 mg/kg of body weight). Thereafter, blood samples were collected into test tubes with heparin through the inferior vena cava and centrifuged, and plasma was separated. Muscle samples (red gastrocnemius muscle with predominant oxidative metabolism and white gastrocnemius muscle with largely anaerobic metabolism) were collected, and visible fatty tissue was mechanically removed. The obtained samples were immediately frozen in liquid nitrogen using precooled aluminum tongs and then stored until further analysis at -80 °C.

5.2 Analysis of plasma glucose and insulin concentrations

Plasma glucose and insulin levels were determined using a Glucose Colorimetric Assay Kit II (BioVision Inc., Milpitas, CA, USA) and Rat Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden), respectively, following the manufacturer's instructions. The intensity of reaction products was measured in a hybrid multimode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA) and, for each measurement, calculated values were based on a separate standard curve. Moreover, the insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), where fasting plasma glucose (FPG) concentration was expressed in millimoles per liter and fasting plasma insulin was expressed in microunits per milliliter (HOMA-IR = (FPG x FPI)/22.5).

5.3 Analysis of sphingolipids content in muscle tissue

Sphingolipids were extracted from the muscle tissue using a mixture of methanol and hydrochloric acid solutions in the presence of internal standards (C17-sphingosine and C17-sphingosine-1-phosphate). The total amount of sphingosine-1-phosphate (S1P) and sphinganine-1-phosphate (SFA1P) was determined by the indirect method after dephosphorylation of sphingosine and sphinganine, respectively, with alkaline phosphatase (ALP; Sigma Aldrich, Saint Louis, MO, USA). The lipid extracts were transferred to a fresh tube with pre-added 40 pmol of N-palmitoyl-d-erythro-sphingosine (C17 base) as an internal standard followed by alkaline hydrolysis to convert ceramide to sphingosine. Afterward, sphinganine (SFA), sphingosine (SFO), dephosphorylated sphingoid bases, and ceramide-derived sphingosine were derivatized. The obtained o-phthalaldehyde derivatives of sphingolipids were analyzed using high-performance

liquid chromatography (HPLC; Varian ProStar, Agilent Technologies, Santa Clara, CA, USA) equipped with a fluorescence detector and C18 reversed-phase column (Varian Inc. OmniSpher 5, 4.6 x 150 mm).

5.4 Analysis of the concentration and fatty acid composition of selected lipid fractions in muscle tissue and plasma

Lipids from the muscle tissue and plasma were extracted with a mixture of chloroform/methanol solution in a volume ratio of 2:1. An internal standard containing heptadecanoic acid, 1,2-diheptadecanoic acid, and triheptadecanoic acid was added to the obtained extracts. Subsequently, the extracts were developed on chromatographic glass plates covered with silica gel and separated into individual lipid fractions: FFA, DAG, TAG, and PL by thin-layer chromatography (TLC) in a heptane/ether isopropyl/acetic acid separation buffer in a volume ratio of 60:40:3. Particular fractions were visualized using a methanolic solution of 2',7'-dichlorofluorescein, identified under UV light on the basis of appropriate standards, and then scraped into separate test tubes. Eluents containing individual lipid fractions were transmethylated in 14% methanolic trifluoride solution and then dissolved in hexane. Particular fatty acid methyl esters (FAME) in each lipid fraction were quantified depending on the retention time of the standards by gas-liquid chromatography (GLC; Hewlett-Packard 5890 Series II gas chromatograph equipped with a HP-INNOWax capillary column and a flame ionization detector, Agilent Technologies, Santa Clara, CA, USA). The total concentrations of lipid fractions were estimated as the sum of individual fatty acid species content within each of the assessed lipid fractions.

5.5 Analysis of the total expression of selected proteins by Western Blot method

In order to determine the total protein expression of proteins directly involved in glucose and sphingolipid metabolism, inflammatory pathway, as well as components of the endocannabinoidome, skeletal muscle samples were homogenized in RIPA buffer containing the cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Manheim, Germany). The bicinchoninic acid method (BCA), with bovine serum albumin (BSA) as a standard, was used to evaluate protein concentration in the homogenates. Subsequently, the homogenates were diluted with Laemmli buffer, and electrophoretic separation of proteins was performed on 10% polyacrylamide gel (CriterionTM TGX Stain-Free Precast Gels, Bio-Rad, Hercules, California, USA). Thereafter, the proteins were transferred onto nitrocellulose or polyvinylidene fluoride

(PVDF) membranes in wet and semi-dry conditions, respectively. Then, to block non-specific bonds, the membranes were incubated in 5% non-fat dry milk or 5% BSA dissolved in Tris-buffered saline with Tween-20 (TBST) buffer and incubated overnight with the appropriate primary antibodies. The next day, the membranes were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology). Afterward, the protein bands were visualized using the chemiluminescence substrate (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA), and then the obtained signals were quantified densitometrically with the use of the ChemiDoc visualization system (Image Laboratory Software Version 6.0.1; Bio-Rad, Warsaw, Poland). The expression of selected proteins was quantified with stain-free gels and the total protein normalization method (Bio-Rad, Hercules, CA, USA).

5.6 Analysis of oxidative stress parameters using commercially available kits

In order to evaluate the oxidative stress parameters, samples of the white and red skeletal muscles were homogenized in RIPA or PBS buffers. The concentrations of selected parameters in skeletal muscle homogenates were determined using commercial colorimetric test kits and ELISA tests. The absorbance of these biomarkers was measured spectrophotometrically using a hybrid multimode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA). Then, from the obtained standard curves, concentrations of oxidative stress markers were calculated according to the manufacturer's protocols.

5.7 Statistical analysis

All the experimental data are presented as mean values ± standard deviation (SD) or percentage of the control group based on six independent determinations. The obtained results were subjected to the Shapiro-Wilk and Barlett's test to assess the distribution of values and the homogeneity of the variance. The statistical differences between the study groups were analyzed using the ANOVA test, followed by the appropriate post hoc test (Tukey's test and t-test). Statistical analysis for all determinations was performed using the GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). The results were considered to be statistically significant at $p < 0.05$.

6. Results

Detailed information on the applied experimental model and obtained research results can be found in the following manuscripts included in the dissertation:

a. Article No. 2 - Bielawiec P., Harasim-Symbor E., Konstantynowicz-Nowicka K., Sztolsztener K., Chabowski A.: *Chronic cannabidiol administration attenuates skeletal muscle de novo ceramide synthesis pathway and related metabolic effects in a rat model of high-fat diet-induced obesity*. Biomolecules, 2020, 10(9),1241, 1-16.
b. Article No. 3 - Bielawiec P., Harasim-Symbor E., Sztolsztener K., Konstantynowicz-Nowicka K., Chabowski A.: *Attenuation of oxidative stress and inflammatory response by chronic cannabidiol administration is associated with improved n-6/n-3 PUFA ratio in the white and red skeletal muscle in a rat model of high-fat diet-induced obesity.* Nutrients, 2021, 13(5), 1604.

The description of the results uses references to figures and tables from the above-mentioned manuscripts included in the dissertation.

In the experimental model of HFD-induced obesity, we noticed a substantial decrease in plasma glucose level in rats fed a standard diet after CBD application (-11.9%, vs. the control group, $p < 0.05$; Table 1, respectively; Article No. 2). Moreover, we observed in both HFD-fed groups (untreated and treated with CBD) a significantly elevated concentration of insulin (+89.9% and +45.2%, vs. the control group, $p < 0.05$; Table 1, respectively; Article No. 2) and significantly increased HOMA-IR index (+59.9% and +39.0%, vs. the control group, $p < 0.05$; Table 1, respectively; Article No. 2). Importantly, we reported that CBD treatment caused a considerable reduction in insulin concentration in the HFD group (-23.5%, vs. the HFD group, $p < 0.05$; Table 1; Article No. 2).

In the case of the sphingolipid pathway, we observed that a HFD caused a substantial intensification of the de novo ceramide synthesis pathway, which resulted in an increase in intramuscular content of SFA, SFA1P, CER and SFO (+21.2%,+231.1%, +25.7% and +14.8%, vs. the control group, $p < 0.05$; Figure 1 A, B, C and D, respectively; Article No. 2). As expected, in the red gastrocnemius muscle chronic CBD treatment of HFD-fed rats significantly reduced the content of the above-mentioned components of the sphingolipid pathway, i.e., SFA, CER and SFO

(–72.9%, -14.9%, -24.3%, vs. the HFD group, $p < 0.05$; Figure 1 A, C and D, respectively; Article No. 2). Only intramuscular content of SFA1P was substantially enhanced after the introduction of CBD in rats fed either the standard chow or the HFD (+306.7% and +325.0%, vs. the control group, $p < 0.05$; Figure 1 B, respectively; Article No. 2). Concomitantly, two-week CBD administration to rats fed the standard diet considerably reduced the content of both SFA and SFO (-53.6% and -26.3%, vs. the control group, $p < 0.05$; Figure 1 A and D, respectively; Article No. 2). Furthermore, we observed significantly decreased intramuscular content of S1P in rats subjected to high-fat feeding (-21.8%, vs. the control group, $p < 0.05$; Figure 1 E; Article No. 2), which was subsequently enhanced by CBD treatment in the same HFD group (+22.4%, vs. the HFD group, $p < 0.05$; Figure 1 E; Article No. 2). Similarly, the value of S1P/CER ratio was restored after CBD introduction in the HFD group (-27.3%, vs. the control group, $p < 0.05$; Figure 1 F; +20.9%, vs. the HFD group, $p < 0.05$; Figure 1 F; Article No. 2).

The induction of obesity by a HFD also induced significant changes in the expression of enzymes involved in the regulation of the sphingolipid pathway. The studies showed significantly increased expression of SPTLC1 and LASS5 (+25.9% and +41.2%, vs. the control group, $p < 0.05$; Figure 2 A and B, respectively; Article No. 2), which was further declined by two-week CBD treatment (-18.2% and -66.4%, vs. the HFD group, $p < 0.05$; Figure 2 A and B, respectively; Article No. 2). Simultaneously, we did not observe any substantial alternations in the total expression of ASAH1 and SPHK2 enzymes ($p > 0.05$; Figure 2 C and D, respectively; Article No. 2) in the HFD group compared to the control conditions. Interestingly, the chronic presence of CBD caused a significant increase in the total expression of the above-mentioned enzymes, i.e., ASAH1 and SPHK2 in the group of rats fed the high-fat diet (+54.6% and +28.7%, vs. the HFD group, $p < 0.05$; Figure 2 C and D, respectively; Article No. 2). Moreover, the opposite effect of CBD was observed in rats subjected to the standard diet, where it reduced the expression of ASAH1 (-47.6%, vs. the control group, $p < 0.05$; Figure 2 C; Article No. 2).

In the skeletal muscle during the course of high-fat feeding, we noticed a considerable decrease in the phosphorylation of proteins involved in the insulin signaling pathway, i.e., IRS-1 and GSK-3 (-13.8% and -24.1%, vs. the control group, $p < 0.05$; Figure 3A and D, respectively; Article No. 2). Concomitantly, two-week CBD administration resulted in a substantial restoration in intramuscular phosphorylation of Akt (Ser-473) and GSK-3 (+59.5% and +38.4%, vs. the HFD group, $p < 0.05$; Figure 3B and D, respectively; Article No. 2). On the other hand, in the case

of glucose transporters GLUT1 and GLUT4, we reported a significant increase in the total muscular expression in the HFD group (+62.9% and +56.4%, vs. the control group, $p < 0.05$; Figure 3 E and F, respectively; Article No. 2), whereas two-week administration of CBD to animals being on a HFD resulted in a considerable reduction in the total expression of both GLUT1 and GLUT4 (-32.9% and -30.8%, vs. the HFD group, $p < 0.05$; Figure 3 E and F, respectively; Article No. 2). The above-mentioned effects of chronic CBD treatment in rats fed a high-fat diet were completed by significantly increased total expression of PDH (+37.9%, vs. the HFD group, $p < 0.05$; Figure 3 G; Article No. 2).

In the case of eCBome components, our study showed that rats subjected to a high-fat diet presented significantly increased total expression of receptors belonging to the ECS system, i.e., CB_1, TRPV1 and 5-HT1A (+47.9%, +61.9% and +93.3%, vs. the control group, $p < 0.05$; Figure 4 A, C and D; respectively; Article No. 2). Unexpectedly, the total muscular expressions of the above-mentioned receptors in HFD groups treated with CBD were considerably decreased (CB_1: -33.9%, TRPV1: -35.4%, 5-HT1A: -62.2%, vs. the HFD group, $p < 0.05$; Figure 4 A, B and D, respectively; Article No. 2). Conversely, the total expression of the CB_2 receptor was markedly enhanced after CBD application in rats fed either standard and high-fat diet (+40.6%, vs. the control group; +43.5%, vs. the HFD group, $p < 0.05$; Figure 4 B, respectively; Article No. 2).

Our study demonstrated that induction of obesity by a high-fat diet caused a relevant reduction of the n-6/n-3 PUFA ratio in the FFA and DAG fractions (-14.2% and -27.6%, vs. the control group, $p < 0.05$; Figure 1 A and B, respectively; Article No. 3) in the white gastrocnemius muscle, whereas two-week CBD treatment considerably enhanced it (+41.7% and +49%.0%, vs. the HFD group, $p < 0.05$; Figure 1 A and B, respectively; Article No. 3). Moreover, in the same muscle type, we also found that two-week CBD injections markedly elevated the n-6/n-3 PUFA ratio during standard and high-fat feeding conditions in the FFA fraction (+11.6% and +21.6%, vs. the control group, $p < 0.05$; Figure 1 A, respectively; Article No. 3). Conversely, in the red gastrocnemius muscle, we observed that high-fat feeding profoundly increased the n-6/n-3 PUFA ratio in the FFA fraction (+12.9%, vs. the control group, $p < 0.05$; Figure 1 A; Article No. 3). In addition, in the same type of skeletal muscle, the chronic presence of CBD caused a significant decrease in the n-6/n-3 PUFA ratio in rats fed either the standard chow or the HFD in the DAG (-6.6% and -13.7%, vs. the control group; -11.6%, vs. the HFD group; $p < 0.05$; Figure 1 B, respectively; Article No. 3) and PL fractions (-21.0% and -21.2%, vs. the control group; -13.1%,

vs. the HFD group; $p < 0.05$; respectively, Figure 1 D; Article No. 3). Regarding the TAG fraction, in both white and red gastrocnemius muscles, we noticed that the rats during high-fat feeding conditions exhibited a markedly elevated n-6/n-3 PUFA ratio (+66.8% and +28.1%, vs. the control group, $p < 0.05$; Figure 1 C, respectively; Article No. 3) with a concomitant reduction of this ratio after a prolonged CBD treatment (-14.9% and -30.1%, vs. the HFD group, $p < 0.05$; Figure 1 C, respectively; Article No. 3). Furthermore, in the TAG fraction, we observed that the n-6/n-3 PUFA ratio was significantly reduced in rats fed a standard chow after CBD application in the white gastrocnemius muscle (-9.5%, vs. the control group, $p < 0.05$; Figure 1 C; Article No. 3) and, conversely, in the HFD-fed rats it was considerably enhanced (+42.0%, vs. the control group, $p < 0.05$; Figure 1 C; Article No. 3). Concomitantly, in the PL fraction, the rats subjected to high-fat feeding exhibited a relevant decrease of the n-6/n-3 PUFA ratio in both the white and red skeletal muscle (-23.8% and -9.3%, vs. the control group, $p < 0.05$; Figure 1 D, respectively; Article No. 3). Moreover, after a prolonged CBD treatment of the HFD-fed rats, we observed a substantially reduced n-6/n-3 PUFA ratio in the PL fraction (-13.9%, vs. the HFD group, $p < 0.05$; Figure 1 D; Article No. 3).

In the plasma, we noticed a considerable increase in the n-6/n-3 PUFA ratio in both HFD-fed groups (untreated and treated with CBD) in the FFA (+47.3% and +37.3%, vs. the control group, $p < 0.05$; Figure 2 A, respectively; Article No. 3) and TAG (+57.1% and +49.1%, vs. the control group, $p < 0.05$; Figure 2 B, respectively; Article No. 3) fractions. Importantly, there was a considerable decline in the n-6/n-3 PUFA ratio in the HFD + CBD group (-6.8%, vs. the HFD group, $p < 0.05$, Figure 2 A; Article No. 3) in the case of the FFA fraction. Concomitantly, in the plasma of the rats fed a standard diet in response to CBD treatment we observed a substantially declined n-6/n-3 PUFA ratio in the FFA and TAG fractions (-7.4% and -6.9%, vs. the control group, $p < 0.05$; Figure 2 A and B, respectively; Article No. 3).

During the course of high-fat feeding, we observed significant alternations of oxidative stress parameters. Regarding CAT concentrations, we noticed a considerable decline in HFD + CBD group only in the red gastrocnemius muscle (-12.8%, vs. the HFD group, $p < 0.05$; Figure 3 A; Article No. 3), whereas we did not observe any changes in catalase values in the white gastrocnemius muscle ($p > 0.05$; Figure 3 A; Article No. 3). Moreover, prolonged CBD treatment during standard and high-fat feeding conditions resulted in a relevant increase in SOD2 levels in the white gastrocnemius muscle (+9.9% and +8.8%, vs. the control group, $p < 0.05$; Figure 3 B,

respectively; Article No. 3). Similarly, in the red gastrocnemius muscle, CBD administration substantially elevated SOD2 concentrations in the rats subjected to standard chow feeding (+7.4%, vs. the control group, $p < 0.05$; Figure 3 B; Article No. 3). Importantly, in both white and red skeletal muscles, two-week CBD treatment resulted in a considerable increase in SOD2 levels in the rats fed the high-fat diet (+7.1% and +3.9%, vs. the HFD group, $p < 0.05$; Figure 3 B; Article No. 3). In relation to the total antioxidant capacity, we noticed a marked elevation in the HFD group after CBD injections only in the white gastrocnemius muscle (+9.1%, vs. the control group, $p < 0.05$; Figure 3 C; Article No. 3). Concomitantly, we did not observe any significant alterations in TAC levels ($p > 0.05$; Figure 3 C; Article No. 3) in the red gastrocnemius muscle. As expected, we noticed significantly elevated AGE levels in both white and red gastrocnemius muscles (+13.3% and +32.1%, vs. the control group, $p < 0.05$; Figure 3 D, respectively; Article No. 3), which was further declined by two-week CBD treatment (-38.8% and -28.0%, vs. the HFD group, $p < 0.05$; Figure 3 D, respectively; Article No. 3). Consequently, the intramuscular MDA concentration was considerably enhanced in the animals being on a high-fat diet in the red skeletal muscle (+45.6%, vs. the control group, $p < 0.05$; Figure 3 E; Article No. 3) with subsequent reduction after administration of CBD (-26.9%, vs. the HFD group, $p < 0.05$; Figure 3 E; Article No. 3). On the other hand, in the white gastrocnemius muscle, the MDA content was markedly diminished after CBD implementation during the course of high-fat feeding (-16.7%, vs. the control group; -22.9%, vs. the HFD group, $p < 0.05$; Figure 3 E; Article No. 3). Moreover, in both white and red skeletal muscles, we observed that high-fat feeding significantly elevated the 4-HNE content (+43.7% and +115.7%, vs. the control group, $p < 0.05$; Figure 3 F, respectively; Article No. 3). Most importantly, after a prolonged CBD treatment of the standard chow-fed rats, we observed a substantial decrease in the 4-HNE concentration in the white skeletal muscle (-39.8%, vs. the control group, $p < 0.05$; Figure 3 F; Article No. 3), while, conversely, we noticed a concomitant increase in its concentration in the same experimental group in the red gastrocnemius muscle (+124.6%, vs. the control group, $p < 0.05$; Figure 3 F; Article No. 3).

As expected, lipid overload conditions significantly alter the total expression of proteins involved in the inflammatory pathway in both muscle types. We observed a pronounced increase in the total intramuscular expression of COX-2 and 5-LO in rats being on a high-fat diet in the red skeletal muscle (+22.3% and +8.9%, vs. the control group, $p < 0.05$; Figure 4 B and C, respectively; Article No. 3). Importantly, the effects of the high-fat feeding in rats were abolished by the two-

week CBD injections in both white and red gastrocnemius muscles, i.e., COX-1 (-49.5% and -39.0%, vs. the HFD group, $p < 0.05$; Figure 4 A, respectively; Article No. 3) and COX-2 (-39.5% and -28.4%, vs. the HFD group, $p < 0.05$; Figure 4 B, respectively; Article No. 3). Moreover, we noticed similar effects of the CBD administration in the rats subjected to high-fat feeding in the case of the total intramuscular expression of 5-LO in the white skeletal muscle (-19.1%, vs. the control group; -26.9%, vs. the HFD group, $p < 0.05$; Figure 4 C; Article No. 3). Simultaneously, after a chronic CBD treatment of the standard diet-fed rats, we observed a markedly diminished total expression of COX-1 and 5-LO in the red skeletal muscle (-32.8% and -17.5%, vs. the control group, $p < 0.05$; Figure 4 A and C, respectively; Article No. 3). Interestingly, CBD treatment of HFD-fed rats enhanced the total expression of anti-inflammatory 15-LO in the white gastrocnemius muscle (+43.2%, vs. the HFD group, $p < 0.05$; Figure 4 D; Article No. 3). Furthermore, in the fatty acids overload conditions the total intramuscular PPARγ expression was considerably decreased in the white and red skeletal muscles (-28.5% and -28.8%, vs. the control group, $p < 0.05$; Figure 4 E, respectively; Article No. 3) and subsequently in the chronic presence of CBD it was restored in both muscle types (+44.2% and +25.0%, vs. the HFD group, $p < 0.05$; Figure 4 E, respectively; Article No. 3). Moreover, in the red gastrocnemius muscle, the rats fed a high-fat diet demonstrated significantly elevated total NF-κB expression (+81.5%, vs. the control group, $p < 0.05$; Figure 4 F; Article No. 3), whereas two-week CBD treatment substantially diminished its muscular expression (-39.4%, vs. the HFD group, $p < 0.05$; Figure 4 F; Article No. 3). Regarding white skeletal muscle, in the case of NF-κB expression we observed similar effects of CBD injections in rats subjected to either standard or high-fat feeding (-27.8% and -35.8%, vs. the control group, $p < 0.05$; respectively; -30.3%, vs. the HFD group, $p < 0.05$; Figure 4 F; Article No. 3). As expected, we reported a pronounced elevation in the total TNF-α expression in the HFD group (+26.6%, vs. the control group, $p < 0.05$; Figure 5 A; Article No. 3), which was subsequently decreased by CBD application (-18.7%, vs. the HFD group, $p < 0.05$; Figure 5 A; Article No. 3) in the white gastrocnemius muscle. Simultaneously, we did not notice any significant alterations in TNF-α expression in the red skeletal muscle ($p > 0.05$; Figure 5 A; Article No. 3). In addition, CBD reduced the total muscular expression of IL-6 during both standard and high-fat feeding conditions in the white gastrocnemius muscle (-34.7% and -39.7%, vs. the control group, $p < 0.05$, respectively; -29.9%, vs. the HFD group, $p < 0.05$; Figure 5 B; Article No. 3). Furthermore, in the course of high-fat feeding, we reported significantly reduced

total expression of Nrf2 in the red gastrocnemius muscle (-25.3%, vs. the control group, $p < 0.05$; Figure 5 C; Article No. 3) and Bcl-2 in both muscle types (-21.5% and -31.3%, vs. the control group, $p < 0.05$, respectively; Figure 5 D; Article No. 3). Interestingly, we noticed a considerable difference in the total muscular expression of Nrf2 in the white gastrocnemius muscle (+30.1%, vs. the control group, $p < 0.05$; Figure 5 C; Article No. 3) and Bcl-2 in the red skeletal muscle (+38.1%, vs. the control group, $p < 0.05$, respectively; Figure 5 D; Article No. 3) in response to CBD treatment during standard feeding conditions. Moreover, prolonged CBD injections markedly increased the total Bcl-2 expression in both white and red gastrocnemius muscle in animals being on a high-fat diet (+29.5% and +50.9%, vs. the HFD group, $p < 0.05$; Figure 5 D, respectively; Article No. 3). Concomitantly, in both muscle types, we noticed that high-fat feeding caused a considerable increase in the total expression of MMP-2 (+37.9%, vs. the control group, $p < 0.05$; Figure 5 E; Article No. 3) and MMP-9 (+19.1% and +27.2%, vs. the control group, $p < 0.05$; Figure 5 F, respectively; Article No. 3). In addition, in the red skeletal muscle of rats during standard or high-fat feeding conditions, we reported a significantly declined the total MMP-2 expression after CBD treatment (-21.6% and -21.8%, vs. the control group, $p < 0.05$, respectively; -33.2%, vs. the HFD group, $p < 0.05$; Figure 5 E; Article No. 3). Most importantly, prolonged application of CBD also noticeably lowered the total expression of MMP-9 in both white and red skeletal muscles (-21.5% and -16.4%, vs. the HFD group, $p < 0.05$; Figure 5 F, respectively; Article No. 3).

7. Conclusions

1. Chronic CBD treatment prevented intramyocellular accumulation of CER (de novo synthesis pathway) and elevated S1P in fatty acids oversupply conditions.
2. CBD improves downstream insulin signaling, mainly by enhancing the phosphorylation ratio of proteins involved in the insulin signal transmission, oxidative metabolism of glucose, and restores glycogen depletion in the myocytes during high-fat feeding.
3. Two-week CBD treatment effectively reduced the accumulation of FAs in the muscular lipid pools and shifted the equilibrium of n-6 to n-3 PUFAs in favor of anti-inflammatory n-3 PUFAs, alleviated inflammation and oxidative stress by reducing the concentration of proinflammatory mediators, e.g., TNF-α and IL-6, the transcriptional activity of NF-κB and AGE and MDA content as well as elevated the antioxidative capacity of the skeletal muscles through increasing the intracellular level of SOD2.

8. Article No. 1

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Phytocannabinoids: useful drugs for the treatment of obesity? Special focus on cannabidiol.

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Phytocannabinoids: Useful Drugs for the Treatment of Obesity? Special Focus on Cannabidiol

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Currently, an increasing number of diseases related to insulin resistance and obesity is an alarming problem worldwide. It is well-known that the above states can lead to the development of type 2 diabetes, hypertension, and cardiovascular diseases. An excessive amount of triacylglycerols (TAGs) in a diet also evokes adipocyte hyperplasia and subsequent accumulation of lipids in peripheral organs (liver, cardiac muscle). Therefore, new therapeutic methods are constantly sought for the prevention, treatment and alleviation of symptoms of the above mentioned diseases. Currently, much attention is paid to *Cannabis* derivatives—phytocannabinoids, which interact with the endocannabinoid system (ECS) constituents. Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are the most abundant compounds of *Cannabis* plants and their therapeutic application has been suggested. CBD is considered as a potential therapeutic agent due to its anti-inflammatory, anti-oxidant, anti-tumor, neuroprotective, and potential anti-obesity properties. Therefore, in this review, we especially highlight pharmacological properties of CBD as well as its impact on obesity in different tissues.

Keywords: cannabidiol, diabetes, drugs, glucose metabolism, obesity, phytocannabinoids

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INTRODUCTION

A well-known ancient plant *Cannabis sativa* has been a subject of scientific interest for over 50 years (1). Moreover, it has been used for recreational and medical purposes for thousands of years. The plant comprises about 100 phytocannabinoids, which are C_{21} terpenophenolic constituents (2). Nowadays, the most-studied phytocannabinoids are: Δ^9- tetrahydrocannabinol (Δ^9-THC), Δ^9-tetrahydrocannabivarin (Δ^9-THCV), cannabinol (CBN), cannabidiol (CBD), cannabidivarin (CBDV), cannabigerol (CBG), and cannabichromene (CBC) (1). So far, many studies have shown therapeutic properties of the above mentioned *Cannabis* compounds. Therefore, the aim of the current review is to focus on the emerging potential of CBD and other phytocannabinoids, which act as novel therapeutic agents in obesity treatment.

THE EXPANDED ENDOCANNABINOID SYSTEM (ECS)

The canonical endocannabinoid system consists of the endocannabinoids (ECs), enzymes responsible for their production and metabolism as well as specific receptors (3). The most studied ECs, i.e., anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are bioactive lipid mediators derived from long-chain polyunsaturated fatty acids (4, 5). It is known that they are released on demand and act through cannabinoid CB_1 and CB_2 receptors, which are G-protein-coupled

receptors (GPCRs) (4–7). Interestingly, in the past the CB_1 receptor was thought to be present only in the brain structures, whereas the CB_2 receptor expression was limited to immune cells (8). However, recent research revealed that both CB_1 and CB_2 receptors are expressed in the brain and peripheral tissues, including the liver, skeletal muscle, heart, gut, bones, and adipose tissue (9–13). ECS mediators, AEA and 2-AG, are degraded by two enzymes: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (14). In addition the existence of many fatty acid-derived mediators, such as *N*-palmitoyl-, *N*-oleoyl-ethanolamine (PEA, OEA), 2-oleoyl-, 2-linoleoyl-glycerol (2-OG, 2-LG), and 2-arachidonoyl glyceryl ether (2-AGE, noladin ether), has been discovered recently (more than 100) (15, 16). These several congeners often share common molecular targets and are inactivated by the same enzymes as ECs. Among these novel targets we include some orphan GPCRs such as GPR55, thermosensitive transient receptor potential (TRP) channels and peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) (17). The aforementioned discoveries have extended the concept of this inner signaling system from ECS itself to the expanded ECS or endocannabinoidome (eCBome). The expanded ECS is involved in controlling various processes, including appetite, energy balance, metabolism, thermogenesis, inflammation, nociception as well as regulation of stress, and emotions (18). Recent data have shown that different phytocannabinoids interact with several components of endocannabinoidome by affecting either cannabinoid receptors (to a lesser extent), non-cannabinoid receptor targets and/or enzymes involved in the metabolism of endogenous ligands (11, 15). Hence, the interest on their regulatory mechanisms and a potent therapeutic action has risen greatly in the last few years.

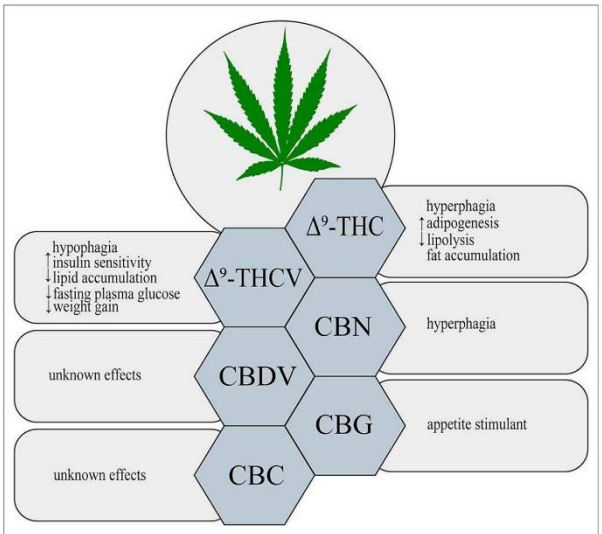

FIGURE 1 | Metabolic effects of different phytocannabinoids. ↑, increase; ↓, decrease; Δ^9-THC, Δ^9- tetrahydrocannabinol; Δ^9-THCV, Δ^9-tetrahydrocannabivarin; CBN, cannabinol; CBDV, cannabidivarin; CBG, cannabigerol; CBC, cannabichromene.

Δ^9-Tetrahydrocannabinol (Δ^9-THC)

Δ^9-THC is a major psychoactive constituent of *Cannabis sativa* (1). It was shown that Δ^9-THC acts as a partial agonist of both CB_1 and CB_2 receptors (19). Throughout such activation Δ^9-THC is able to trigger many different physiological processes, i.e., regulation of gastrointestinal, liver and cardiovascular functions, pain perception along with modulation of neurotransmitters release in the nervous system (20, 21). Now, it is clearly established that, Δ^9-THC exerts a well-known psychoactive effect, which is mediated by the activation of CB_1 receptor in the central nervous system (CNS) (22). Additionally, Δ^9-THC by activating CB_1 receptors, located in the limbic (enhancing motivational properties of food) and hypothalamic (increasing appetite) structures, causes the orexigenic effects (**Figure 1**) (23). Apart from the above implications, Δ^9-THC has also the ability to bind to eCBome receptors including GPR55 (G protein-coupled receptor 55), 5- HT_{3A} (serotonin receptor subunit), TRPV2, 3, 4 receptors (transient receptor potential channels of vanilloid type 2, 3, 4), which produce some of its pharmacological effects (22). Lauckner et al. revealed that Δ^9-THC activates the GPR55 receptor in HEK293 and CHO cells, which resulted in increased intracellular calcium level (24). Another study conducted on HEK293 cells showed that Δ^9-THC acts as a 5- HT_{3A} receptor antagonist, whereas several reports demonstrated that Δ^9-THC has agonistic effects on the TRPV2, 3, 4 channels (25–28). Furthermore, Δ^9-THC was reported to exhibit a beneficial impact on the regulation of insulin sensitivity in the insulin resistant adipocytes. The authors have demonstrated that natural extract containing Δ^9-THC decreased the TAGs content and improved the glucose uptake in the insulin resistant 3T3-L1 cells in a concentration-dependent manner (29). In the same study it was shown that the level of tumor necrosis factor alpha (TNF-α) in the 3T3-L1 cells was substantially decreased in the presence of Δ^9-THC, which also improved sensitivity of the cells to insulin (29). The above mentioned studies also revealed that in a differentiated 3T3-L1 cells Δ^9-THC treatment enhanced glucose transporter type 4 (GLUT4) and insulin receptor substrate 1 and 2 (IRS-2) gene expressions, which play key roles in the insulin signaling pathway (29). However, we have to keep in mind that in the above experimental models natural extract were applied and no information upon their purity was provided, therefore, it can be considered as the limitation of such studies in the face of stating pharmacological properties of examined substances.

On the other hand, in comparison to the well-documented orexigenic effect of Δ^9-THC, many studies have also shown a potential anti-obesity effect of this phytocannabinoid derivative. Cluny et al. (30) using the *in vivo* model have demonstrated that chronic administration of Δ^9-THC to diet-induced obesity (DIO) mice for 3 and 4 weeks treatment (intraperitoneal injections of Δ^9-THC in a dose of 2 or 4 mg/kg and vehicle) prevented weight gain due to reduced energy intake (30). This effect might have been associated with the fact that Δ^9-THC is a partial agonist of CB_1 and CB_2 receptors. Since, Δ^9-THC does not produce maximal stimulation of the above receptors, the background of such changes could result from blocking

endogenous full agonist, i.e., anandamide from binding to CB_1 receptor, especially, when the endocannabinoid tone is high (e.g., obesity) (30, 31). However, potential positive influence of Δ^9-THC in the prevention and treatment of obesity requires further investigation.

Δ^9-Tetrahydrocannabivarin (Δ^9-THCV)

Δ^9-THCV is a propyl Δ^9-THC analog, which binds to the cannabinoid CB_1 and CB_2 receptors (32). So far, data indicate that Δ^9-THCV is a neutral antagonist at low doses and agonist at high doses of CB_1 and CB_2 receptors (2, 32, 33). Little evidence exists upon the activation of various eCBome receptors by Δ^9-THCV. Nevertheless, it has been established that Δ^9-THCV acts as an agonist of human TRPV1, rat TRP channels of ankyrin type-1 (TRPA1) and TRPV2, 3, 4 channels as well as an antagonist of rat's TRP channel of melastatin type-8 (TRPM8) (26). In one study it was demonstrated that Δ^9-THCV activated 5-HT_{1A} receptor in the rat brain and human 5-HT_{1A} receptor-transfected CHO (Chinese hamster ovary) cells, and thereby exerted antipsychotic effects (34). Interestingly, recent research has shown that this non-psychotropic phytocannabinoid has a potential therapeutic effect in the treatment of diabetes associated with obesity. Wargent et al. (35) revealed that Δ^9-THCV increased insulin sensitivity and improved glucose tolerance in DIO mice as well as genetically obese *ob/ob* mice (**Figure 1**). However, it did not substantially affect food intake and body weight gain in the above models, whereas body fat content was decreased (35). In the same experiment, the authors investigated the impact of Δ^9-THCV on the insulin resistant C_2C_{12} myotubes and HL-5 hepatocytes showing that Δ^9-THCV restored intracellular insulin signaling pathway (35). Other studies have also displayed that Δ^9-THCV (1–10 μM) decreased lipid accumulation in the *in vitro* model of hepatosteatosis in HHL-5 (Human Hepatocyte Line 5) cells and adipocytes (3T3-L1 cells) (36). According to the above results, a pilot study in patients with type 2 diabetes showed that Δ^9-THCV reduced fasting plasma glucose with parallel improvement in β-cell function as well as increased Apo A (apolipoprotein A) and adiponectin concentrations compared with placebo, which was well-tolerated in patients (37). Nonetheless, further research is necessary to confirm the potential therapeutic properties of Δ^9-THCV in the treatment of obesity, metabolic syndrome and type 2 diabetes.

Cannabinol (CBN)

An oxidized metabolite of Δ^9-THC, cannabinol, was isolated in 1896 by Wood and colleagues (38). CBN is a non-enzymatic oxidative breakdown product of Δ^9-THC due to aging or light exposure (32). It was shown that CBN binds to cannabinoid receptors CB_1 and CB_2 in the peripheral organs and central nervous system (CNS), and therefore, it exerts a weak psychoactive activity (39). CBN also displays the ability to bind to expanded ECS receptors including agonistic and antagonistic effects on TRPA1 and TRPM8 channels, respectively (26). So far, studies focusing on the impact of CBN on obesity and feeding behavior have been limited. Only in one of the recent studies it has been demonstrated that CBN increased food intake in rats in a dose-dependent manner (**Figure 1**) (40). Probably, the hyperphagic CBN's effect was induced by its interaction with CB_1 receptor in CNS (40) but it should be confirmed by additional experiments including for example gene silencing or knockout animals. Nevertheless, further experiments are required to completely characterize the role of CBN in food consumption and body weight control.

Cannabidivarin (CBDV)

Among phytocannabinoids we can find a propyl analog of CBD, cannabidivarin, which exerts non-psychotropic properties (32). CBDV has a weak affinity for cannabinoid receptors CB_1 and CB_2 with simultaneous stronger activation of other molecular targets related with the ECS (41). Predominantly, anticonvulsant and other therapeutic CBDV's properties are associated with activation of TRP channels (TRPV1, 2 and TRPA1) and TRPM8 inhibition (26, 42). Another pharmacological target for CBDV is GPR55 receptor, where observed effect is inhibitory (43). However, currently there is insufficient research on the ground of CBDV's effects on obesity, insulin resistance and other metabolic disturbances (**Figure 1**).

Cannabigerol (CBG)

Another phytocannabinoid, which lacks psychotropic properties and occurs only in trace amounts in *Cannabis*, is cannabigerol (41). *Cannabis* plants produce cannabigerolic acid (CBGA), which is a direct precursor of cannabinoids: Δ^9-THC, CBC and CBD (44, 45). Preliminary studies, using plasma membranes isolated from the CHO and HEK-293 cells expressing human CB_1 and CB_2 receptors, have shown that CBG acts as a partial agonist of both cannabinoid receptors (46). Similarly to other phytocannabinoids CBG has an ability to interact with endocannabinoidome receptors, such as TRPV1, 2, TRPA1, and TRPM8 channels (26). Moreover, one study has provided for the first time evidence upon activation of α_2-adrenoreceptors and 5-HT_{1A} receptor blockage by CBG in the vas deferens and brain, respectively (47). Furthermore, recent investigation has revealed that CBG serves as a novel appetite stimulant in rats and importantly, no side effects were observed during its administration (**Figure 1**) (48). However, extensive research is necessary to determine a more detailed mechanisms of CBG action with respect to metabolic diseases.

Cannabichromene (CBC)

The discovery of cannabichromene was reported by Izzo et al. (32). It is a minor component of *Cannabis* plant, which presents low affinity for CB_1 and CB_2 receptors (49, 50). Nonetheless, researchers discovered another molecular target of CBC, i.e., TRP channels. De Petrocellis et al. revealed that CBC is the most potent agonist of the TRPA1 channels (26). However, it has also the ability to activate in a lower potency, TRPV3 and TRPV4 channels, and additionally inhibits TRPM8 receptors, but to a much lesser extent (26). Although CBC exhibits different medical properties, its influence on metabolism and obesity has not been found, yet (**Figure 1**).

Cannabidiol (CBD)

Cannabidiol (CBD) is one of the most common non-psychotropic constituent of *Cannabis* plant (this depends on the cannabis strain). It was revealed that CBD induces a wide-range of pharmacological effects through different mechanisms. CBD functions as a negative allosteric modulator of CB_1 receptor (51). Therefore, it has therapeutic potential in the treatment of the central nervous system diseases (neurodegenerative diseases, epilepsy, anxiety, and depression) without concurrent psychotic side effects (2, 52, 53). Furthermore, it was indicated that CBD is able to block CB_1 receptor, thereby producing anti-obesity effects. Otherwise, CBD unexpectedly exhibited a high affinity for CB_2 receptor as an agonist or inverse agonist depending on the research model (*in vitro* or *in vivo*) (54, 55). An interesting result of the latest studies was the fact that CBD has greater affinity for various eCBome receptors, including GPR55, α1-adrenoreceptors, 5-HT_{1A}, TRPV channels and PPARγ (**Figure 2**) (19, 26, 42, 56, 57). Hegde et al. as well as Esposito et al. reported that CBD significantly induced the transcriptional activity of PPARγ (58, 59). It is known that PPARγ is an interesting therapeutic target due to its crucial role in regulating glucose homeostasis, lipoprotein metabolism, and inflammation (60, 61).

Moreover, recent research has focused on the therapeutic properties of CBD, including anti-inflammatory, anti-oxidant, anti-tumor, anti-convulsant and neuroprotective effects (62, 63). In the light of the above mentioned properties, CBD emerges as a potential therapeutic agent, which can be used in the treatment of diabetes and its complications, obesity, ischemia, neurodegenerative diseases as well as pain relieving and depression. Although CBD's anti-inflammatory and neuroprotective properties are well-confirmed, there are few studies that have investigated anti-obesity effects of this compound. Wierucka-Rybak et al. have examined the effect of CBD on food intake, food preferences and weight gain in rats (64). The authors administered CBD (3 mg/kg) for 3 days to rats maintained on a standard diet (SD), high fat diet (HFD), or free choice diet (FC; high sucrose). The authors revealed that CBD injections in the case of rats being on the HFD resulted in an increase in body weight despite significantly reduced food intake. On the other hand, in rats fed FC diet CBD did not cause significant change in food consumption and body weight (64). Previous studies investigating the impact of CBD on food intake showed contradictory results. One study demonstrated CBD-induced (2.5 and 5 mg/kg) decrease in body weight gain in rats (54), while other studies have shown no significant impact on food intake and body weight in mice and rats (65, 66). Importantly, pilot studies were conducted to investigate the effect of CBD on glycemic and lipid parameters in patients with type

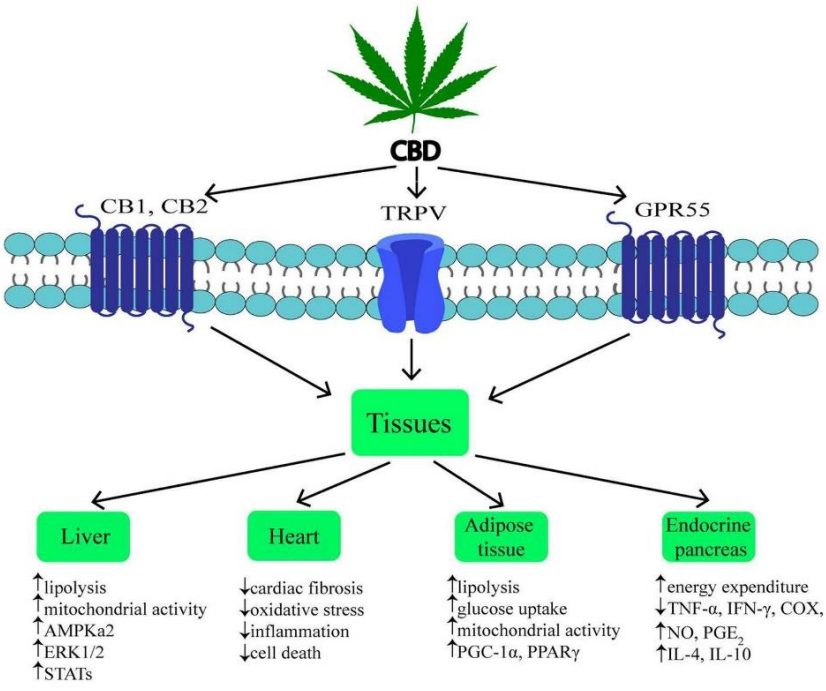

FIGURE 2 | Mechanism and effects of CBD action on various tissues during obesity. ↑, increase; ↓, decrease; CBD, cannabidiol; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; TRPV1, transient receptor potential channel of vanilloid type 1; GPR55, G protein coupled receptor 55; AMPKa2, catalytic subunit of AMP-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; STATs, signal transducers and activators of transcription; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARγ, peroxisome proliferator-activated receptor gamma; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; COX, cyclooxygenase; NO, nitrogen oxide; PGE_2, prostaglandin E2; IL-4, interleukin 4; IL-10, interleukin 10.

2 diabetes (37). The findings from these studies demonstrated that CBD did not produce any improvement in glycemic and lipid control despite producing eligible changes in gut hormones (GIP—glucose dependent insulinotropic peptide) and adipokines (resistin) concentrations (37). Probably, the reason for the lack of therapeutic effects seen during CBD administration could have been too low dosage used in the study. The anti-epileptic properties of CBD were investigated in many preclinical studies and in randomized trials (67–71). Data obtained from these studies confirmed CBD's anti-seizure properties along with a good tolerance profile. Currently, the first cannabis based medicine containing CBD/Δ^9-THC combination for the multiple sclerosis related spasticity treatment (Sativex/Nabiximols; GW Pharmaceuticals) has been approved in numerous countries, including Australia, Canada, New Zealand and in most European Union countries (72). Furthermore, a drug composed solely of CBD (Epidiolex; GW Pharmaceuticals) has been approved in June 2018 in the United States for the treatment of Dravet and Lennox-Gastaut syndromes in children (52, 73, 74).

OVERACTIVATION OF THE ECS IN OBESITY

Obesity and coexisting disorders such as insulin resistance, hypertension, and hypertriglyceridemia lead to the development of metabolic syndrome and type 2 diabetes (75). Whenever, the excessive fatty acids (FAs) consumption takes place, simultaneously we can observe an increased differentiation of pre-adipocytes to mature adipocytes with subsequent stimulation of their growth as well (76, 77). Over time, at a later stage of obesity development, adipocytes are overloaded, which results in the accumulation of lipids in other tissues such as liver, skeletal, and cardiac muscles (60, 78). Paralell excessive fat accumulation can be observed in the liver or cardiac muscle, which contributes to the development of liver steatosis and cardiomyopathy, respectively (78). Many scientists are looking for new therapeutic strategies, including expanded ECS, which can be a useful tool in preventing and treating the above mentioned diseases. Therefore, the components of eCBome are emerging as potent therapeutic targets due to their well-established role in the regulation of food consumption and energy balance as well as lipid and glucose metabolism (79, 80). Literature data indicated, that ECS is upregulated during obesity and associated diseases (81–84). It is well-confirmed that the level of endogenous cannabinoids in the above mentioned conditions is increased, i.e., in CNS, adipose tissue, pancreas, skeletal muscle, kidney, liver, and blood of obese rodents and humans (76, 84–87). The cause of such ECS overactivation may be due to enhanced synthesis of ECs or their reduced degradation as well as overexpression of the cannabinoid receptors (88, 89). Various studies have shown upregulated levels of 2-AG in both different organs and serum during obesity and hyperglycemia, which was correlated with body fat content, visceral fat mass and fasting plasma triacylglyceride and insulin concentrations (84, 86, 87, 90). On the other hand, the reverse situation was described for ECs in the liver of DIO mice, where the hepatic levels of AEA were increased in animals fed HFD, while no significant difference in 2-AG liver levels was observed (91). Accordingly, Kimberly et al. revealed substantial association between AEA level and body mass index (BMI) value, which was an argument for making it a biomarker of NASH (non-alcoholic steatohepatitis) (92). In contrast with the above results, other studies have shown that patients with NAFLD (non-alcoholic fatty liver disease) had significantly increased levels of 2-AG without any change in AEA levels (93). Hence, it has been proposed to attenuate overactivation of ECS as a new approach for the treatment of obesity and its coexisting disorders. Such mechanism was used by researchers to create an anti-obesity drug (rimonabant; SR141716A), which was the first selective antagonist of CB_1 receptors expressed in the brain and different peripheral organs/tissues controlling energetic homeostasis of the body (liver, muscle, adipose tissue, etc.) (94–96). Several studies have confirmed the beneficial effect of rimonabant on cardiometabolic risk markers, body weight as well as lipid and glucose parameters (97–99). However, rimonabant (Acomplia®; Sanofi-Aventis) treatment turned out to be harmful so that it was forbidden in 2009 due to its adverse psychotropic side effects (100). This contributed to the invention of peripherally restricted CB_1 receptor antagonists with limited brain penetration. Many studies investigating the effects of these antagonists (inverse agonists), such as AM6545, JD5037, have shown their positive effects against obesity in preclinical studies (101–103). For instance, AM6545 (10 mg/kg per day) treatment in DIO and genetically obese (*ob/ob*) mice attenuated obesity-related glucose intolerance, insulin resistance, dyslipidemia and reversed hepatic steatosis (104). Accordingly, Tam et al. demonstrated the hypophagic and weight-reducing effects of JD5037 (3 mg/kg per day, 7 days) in DIO mice but not in *ob/ob* and *db/db* mice, indicating leptin-dependent action (102). Importantly, JD5037 treatment resulted in the attenuation of hyperglycemia, hyperinsulinemia, insulin resistance, and reduction of hepatic triacylglycerols in all the above mentioned strains (102). We can conclude that peripherally restricted CB_1 receptor antagonists have great therapeutic potential in the treatment of obesity.

SPECIFIC SITES OF CBD ACTIONS RELATED TO OBESITY

Liver

In view of the increasing prevalence of obesity, type 2 diabetes and metabolic syndrome, the development of liver diseases occurs more often. The above mentioned diseases are associated with excessive fat accumulation resulting from upregulated FA influx and *de novo* lipid synthesis in different cells, e.g., hepatocytes (105). Alterations in the hepatic fatty acid oxidation are highly related with the aforementioned metabolic disturbances, which were shown in many experimental models together with discrepancies in this field. Studies conducted on NASH and liver steatosis patients showed an increase (106, 107), decrease (108), or no change (109) in the fatty acid oxidation

status. On the other hand, the expected consequence of lipid accumulation in the liver would be simultaneous enhancement in hepatic oxidation of fatty acids, thus, it seems that certain factors can also influence this process as well.

In previous studies it was shown that, hepatocytes produce endocannabinoids, AEA and 2-AG, and possess CB_1 and CB_2 receptors (80, 91). A study carried out by Liu et al. revealed that liver specific CB_1 receptor knockout mice were protected from the development of both insulin resistance and hepatosteatosis but not obesity (55). Silvestri et al. revealed positive influence of CBD on the liver, namely the reduction of intracellular lipid content in the *in vitro* hepatosteatosis model, possibly by enhancing lipolysis and mitochondrial activity through increased fatty acids oxidation (36). The authors have shown that CBD increases the expression of selected proteins involved in upregulating lipid metabolism, i.e., catalytic subunit of 5′AMP-activated protein kinase (AMPKa2), extracellular signal-regulated kinase (ERK1/2) along with signal transducers and transcription activators (STATs) in hepatocytes (**Figure 2**, **Table 1**) (36). Concomitantly, the authors observed that CBD enhanced the level of glutathione (GSH), adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NAD), which supported the assumption that CBD increases intracellular lipolysis and mitochondrial activity (36). Additionally, the study carried out by Wang's research group (119) indicated, in a murine model, an impact of CBD on alcohol-induced liver injury. The obtained results displayed that CBD attenuates liver steatosis (decreased triacylglyceride and fat droplet accumulation), inflammatory response [reduced the alcohol-feeding induced mRNA expressions of interleukin 1beta (IL1β), TNF-α and monocyte chemoattractant protein 1 (MCP1)], oxidative/nitrative stress (reduced lipid peroxidation, expression of reactive oxygen species generating enzyme—NADPH oxidase 2 (NOX2) and 3-nitrotyrosine production) and neutrophil infiltration in the liver (**Table 1**). This also confirmed the well-known, whole body anti-inflammatory and antioxidant properties of CBD (119).

Adipose Tissue

Obesity is associated with chronic low-grade inflammatory state and excessive fat accumulation (120). The expression of CB_1 and CB_2 receptors, other eCBome molecular targets

TABLE 1 | Summary of the effects of CBD on different tissues during obesity.

Organ	ECS overactivation	CBD treatment	References
Brain	↑food intake, ↑motivation for palatable food	Unknown	(9, 23)
Liver	↑lipogenesis, ↑fibrogenesis, ↑hepatic apoptosis, ↑hepatocyte proliferation, ↓AMPK, ↑dyslipidemia, ↑steatosis, ↑fibrosis, ↑insulin resistance	↓liver enzymes level, ↓steatosis, ↓inflammation, ↓liver damage, ↓pro-inflammatory cytokines	(83, 110–112)
Gastrointestinal tract	↑food assimilation, ↑permeability, ↓satiety, ↓motility, ↓inflammation	↑GIP level, ↓resistin concentration	(37, 110, 111)
Adipose tissue	↑lipogenesis, ↑glucose uptake, ↓FA oxidation, ↓lipolysis, ↓mitochondrial biogenesis, ↓UCP1, ↑storing capacity, ↑insulin resistance, ↓thermogenesis	↑lipolysis, ↑thermogenesis, ↑PGC-1α, UCP1, PPARγ expression, ↓lipogenesis	(31, 113)
Endocrine pancreas	↑/↓insulin secretion, ↑insulin resistance, ↑Bad protein activation, ↑β-cell death	↑anti-inflammatory cytokines ↓pro-inflammatory cytokines	(114–116)
Muscles	↓insulin signaling ↓glucose uptake, ↓AMPK	Unknown	(9, 83)
Heart	↑AT1 receptor expression, ↑ROS generation, ↑MAPK activation, ↑AGEs accumulation ↑hypotension, bradycardia, negative inotropy, ↑inflammation, ↑apoptosis, ↓inflammatory response	↓infarct size, ↓infiltrating leukocytes, ↓platelet aggregation, ↓inflammation, ↓fibrosis	(11, 117, 118)

↑, increase; ↓, decrease; CBD, cannabidiol; AMPK, AMP-activated protein kinase; GIP, glucose dependent insulinotropic peptide; UCP1, uncoupling protein 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPARγ, peroxisome proliferator-activated receptor gamma; AT1, angiotensin II receptor type 1; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; AGEs, advanced glycation end products.

(i.e., TRPV1,GPR55) and ECs enzymes level in the visceral and subcutaneous adipose tissue has been found (121, 122). These additional receptors are the most promising target for major non-psychogenic constituent of *Cannabis plant*, which has well-established anti-inflammatory effects and potential anti-obesity properties (123). Silvestri et al. (36) pointed out that CBD, depending on the time and dose, reduced triacylglycerols accumulation in 3T3-L1 adipocytes treated with oleic acid (OA). These results emphasize a potential role of CBD on lipolysis induction (**Table 1**). However, more research is necessary to investigate the exact mechanism of this phenomenon (36). Similarly, Ramlugon et al. (124) revealed that CBD treatment, in a time-dependent manner, induced the mitochondrial activation and increased oxygen consumption, which may be an explanation for the reduced fat accumulation in adipocytes despite of increased glucose uptake (**Figure 2**) (124). Additionally, recent research has shown that CBD inhibited weight gain in rats subjected to high fat diet (HFD) for 14 days and this effect was probably mediated by CB_2 receptor (54). The authors confirmed the above effect by using a selective CB_2 antagonist-AM630, which prevented the reduction in weight gain due to CBD treatment (54). However, additional studies are required to uncover the mechanisms by which CBD induces the above metabolic changes in adipocytes.

Endocrine Pancreas

The pancreas plays a pivotal role in the blood glucose homeostasis and whole body metabolism through insulin and glucagon secretion (125). In obesity, secretion of the above hormones is dysregulated. To date, the expression of cannabinoid receptors within the endocrine pancreas has been shown (114, 121). However, inconsistent data have been obtained regarding the expression of CB_1 and CB_2 receptors in the pancreas islet cells. Starowicz et al. (121) demonstrated the expression of CB_1 receptor in glucagon-secreting α-cells of mice, while CB_2 receptor was found inside mouse α-cells and insulin-secreting β-cells. Similarly, in the study conducted on human samples it was determined that CB_1 receptor was present in glucagon-secreting α-cells and in a lesser extent in insulin-secreting β-cells, whereas CB_2 was only located in somatostatin-secreting delta cells (114). On the other hand, other studies indicated that β-cells exhibited the expression of both cannabinoid receptors or only CB_2 receptor (84, 115). Interestingly, Levendal et al. (126) investigated the effect of CBD on β-cell function in obese rats. The authors found that in a rat model of diet-induced obesity (DIO), *cannabis* extract treatment reduced the weight gain (mainly fat depots) and increased energy expenditure through upregulation of protein kinase B (PKB), mitochondrial uncoupling protein 2 (UCP2), and glucose transporter 2 (GLUT2) expression in pancreatic β-cells (rats were injected subcutaneously every second day for 28 days; the first five treatments containing an equivalence of 5 mg THC/kg body weight and the remaining treatments an equivalence of 2,5 mg THC/kg body weight) (126). Moreover, studies conducted by Weiss et al. have shown that CBD treatment (mice were administered 10–20 intraperitoneal injections at a dose of 5 mg/kg CBD) decreased the frequency of type 1 diabetes incidence and development in a model of non-obese diabetic (NOD) mice (116). Furthermore, the authors reported that CBD reduced plasma level of the pro-inflammatory cytokines, i.e., TNF-α and interferon gamma (IFN-γ) together with inflammation mediators, such as nitric oxide (NO), cyclooxygenase (COX), and prostaglandin E2 (PGE2). Whereas, anti-inflammatory cytokines—interleukins IL-4, IL-10 levels were increased (**Figure 2**, **Table 1**). Hence, reduced pancreas islets inflammation was observed in the histological examination as well as lower β-cell destruction (116). Concluding, it is likely that CBD has the ability to prevent and reduce the pancreatic damage associated with obesity and insulin resistance.

Cardiac Muscle

Chronic inflammatory state associated with obesity reduces the ability of the adipose tissue to store incoming plasma-borne fatty acids and leads to their accumulation in other organs, including cardiac muscle (127). Then, the local inflammatory state, oxidative stress and hyperinsulinemia can develop and progress induction of insulin resistance in the heart muscle (128). Currently, many studies are focused on the effect of CBD on the lipid and glucose metabolism in the heart, which is dysregulated during obesity and subsequently can lead to the cardiomyopathy and cardiac contractile dysfunction development (117). The impact of CBD on myocardial dysfunction, oxidative/nitrative stress, inflammation, and cell death has been recently investigated by Rajesh and coworkers (117). They used a primary human cardiomyocytes subjected to a high glucose concentration and a mouse model of type 1 diabetic cardiomyopathy. It was demonstrated that CBD reduced cardiac fibrosis, myocardial oxidative/ nitrative stress, inflammation and cell death in diabetic hearts (in the first set of experiments, 1 week diabetic mice were treated with CBD 1, 10 or 20 mg/kg intraperitoneal injections for 11 weeks; in the second set of experiments 8 weeks diabetic mice were treated with CBD for 4 weeks) (**Figrue 2**, **Table 1**) (117). These effects were mediated by decreased intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and TNF-α expression as well as reduced mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NFκB) activation. Moreover, CBD attenuated the high glucose-induced enhanced ROS (reactive oxygen species) generation and cell death in human cardiomyocytes (117). These findings highlight the role of CBD in the prevention or treatment of diabetic complications.

CONCLUSIONS

Overweight, insulin resistance and obesity emerged as leading health concerns all over the world. The above mentioned disturbances are characterized by excessive or abnormal fat accumulation, and are major risk factors for a number of chronic diseases, such as cardiovascular diseases, diabetes, and cancer. Currently, the non-psychotropic component of *Cannabis sativa*—CBD is in the center of interest, due to its well-established anti-inflammatory, anti-oxidant, anti-convulsant, anti-psychotic and potential anti-obesity properties. Many studies indicated that CBD affects both lipid and glucose metabolism through the action on various receptors as well as several metabolites. From the existing data, we can conclude that CBD has the

promising potential as a therapeutic agent and might be effective in alleviating the symptoms of insulin resistance, type 2 diabetes and metabolic syndrome.

AUTHOR CONTRIBUTIONS

PB participated in the design of the work, drafted the manuscript, prepared figures and tables, and approved final version submitted. EH-S helped to draft the manuscript and approved final version submitted. AC participated in the design of the study, revised manuscript, and approved final version submitted. All authors agreed to be accountable for all aspects of the work.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Article No. 2

Patrycja Bielawiec, Ewa Harasim-Symbor, Karolina Konstantynowicz-Nowicka, Klaudia Sztolsztener, Adrian Chabowski

Chronic cannabidiol administration attenuates skeletal muscle de novo ceramide synthesis pathway and related metabolic effects in a rat model of high-fat diet-induced obesity.

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Chronic Cannabidiol Administration Attenuates Skeletal Muscle De Novo Ceramide Synthesis Pathway and Related Metabolic Effects in a Rat Model of High-Fat Diet-Induced Obesity

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Abstract: Numerous studies showed that sustained obesity results in accumulation of bioactive lipid derivatives in several tissues, including skeletal muscle, which further contributes to the development of metabolic disturbances and insulin resistance (IR). The latest data indicate that a potential factor regulating lipid and glucose metabolism is a phytocannabinoid—cannabidiol (CBD), a component of medical marijuana (*Cannabis*). Therefore, we aimed to investigate whether chronic CBD administration influences bioactive lipid content (e.g., ceramide (CER)), as well as glucose metabolism, in the red skeletal muscle (musculus gastrocnemius) with predominant oxidative metabolism. All experiments were conducted on an animal model of obesity, i.e., Wistar rats fed a high-fat diet (HFD) or standard rodent chow, and subsequently injected with CBD in a dose of 10 mg/kg or its solvent for two weeks. The sphingolipid content was assessed using high-performance liquid chromatography (HPLC), while, in order to determine insulin and glucose concentrations, immunoenzymatic and colorimetric methods were used. The protein expression from sphingolipid and insulin signaling pathways, as well as endocannabinoidome components, was evaluated by immunoblotting. Unexpectedly, our experimental model revealed that the significantly intensified intramuscular de novo CER synthesis pathway in the HFD group was attenuated by chronic CBD treatment. Additionally, due to CBD administration, the content of other sphingolipid derivatives, i.e., sphingosine-1-phosphate (S1P) was restored in the high-fat feeding state, which coincided with an improvement in skeletal muscle insulin signal transduction and glycogen recovery.

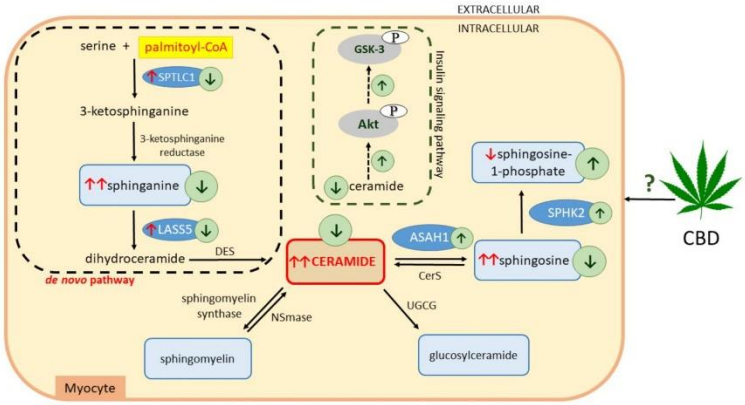
Keywords: cannabidiol; obesity; insulin resistance; ceramide; sphingolipids; glucose; insulin signaling

1. Introduction

Currently, obesity is a widespread medical condition reaching high rates in children and adults. Since 1975, the worldwide incidence of obesity increased almost threefold according to the World Health Organization [1]. The majority of obesity cases is the consequence of excessive food consumption and a sedentary lifestyle [2]. Sustained obesity disrupts metabolic processes and pathways, especially glucose and fatty acid (FA) metabolism, which is the background of lipotoxicity and insulin resistance (IR) [3], leading to the further development of metabolic syndrome (MetS) and type 2 diabetes mellitus (T2D) [4].

An increased intake of fatty acids in a diet at an advanced stage of obesity progression results in adipocyte overload and the abnormal accumulation of bioactive lipid fractions in several tissues,

including skeletal and cardiac muscle, as well as the liver [5,6]. Over the last several years, attention was paid to sphingolipids, the synthesis of which is increased during the overfeeding state (Scheme 1) [7]. Importantly, recent data clearly demonstrated that sphingolipids are not only structural components of the cells; they also act as signaling molecules participating in growth regulation, cell differentiation, apoptosis, and signal transduction [8]. It was shown that intramyocellular lipids, especially ceramide (CER), directly interfered with the insulin transduction signal pathway in the target tissues [5], which subsequently resulted in a deterioration of insulin-stimulated glucose uptake [9]. This seems to be of great importance since skeletal muscle, due to its mass, significantly contributes to the overall energy expenditure, for instance, by being responsible for nearly 80% of postprandial glucose uptake [9].



Scheme 1. Effects of a high-fat diet (HFD) and chronic cannabidiol (CBD) administration on the sphingolipid metabolic pathway in rat myocytes. ↑, increase; ↓, decrease; red arrows indicate the effects of high-fat feeding; green arrows indicate the effects of CBD treatment; serine palmitoyltransferase, long chain base subunit 1 (SPTLC1); ceramide synthase 5 (LASS5); dihydroceramide desaturase (DES); neutral sphingomyelinase (NSmase); UDP-glucose ceramide glucosyltransferase (UGCG); ceramide synthase (CerS); acid ceramidase (ASAH1); sphingosine kinase 2 (SPHK2); phosphorylated protein kinase B (pAkt); phosphorylated glycogen synthase kinase 3 (pGSK-3).

Currently, a new therapeutic approach is being sought for the prevention and treatment of obesity and coexisting disorders. The endocannabinoid system (ECS) was in the spotlight for several decades due to its well-established role in the regulation of appetite and energy expenditure [10,11]. It consists of cannabinoid receptors, CB1 and CB2, which are widespread throughout the body and located both in the central nervous system (CNS) and in the peripheral tissues (e.g., skeletal muscle, liver, adipose tissue) [12–14]. Cannabinoid receptors are sensitive to endogenous ligands (endocannabinoids (ECs)), mainly *N*-arachidonoylethanolamine (anandamide (AEA)) and 2-arachidonoylglycerol (2-AG), which are long-chain polyunsaturated fatty-acid derivatives [15]. The ECs, AEA and 2-AG, have their own metabolic routes, including enzymes responsible for their degradation: fatty acid amid hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively [16]. Recently, new fatty-acid derivatives (more than 100), along with their corresponding molecular targets, were discovered, among which certain orphan G-protein-coupled receptors (GPCRs) like GPR55, transient receptor potential (TRP) channels like the TRP of vanilloid type-1 (TRPV1), and peroxisome proliferator activated receptors α and γ (PPARα and PPARγ) are present [17]. The above-mentioned components, cannabinoid and non-cannabinoid receptors, lipid mediators, and specific enzymes, based on recent findings, form the expanded ECS or endocannabinoidome (eCBome), which is an extension of the classic definition of the ECS [18].

Numerous studies showed that, during obesity, the ECS is overactivated; therefore, it emerges as a promising target in the treatment of obesity with a considerable physiological significance [19–21]. It was soon realized that cannabidiol (CBD) is a potential therapeutic agent on the grounds of its well-confirmed anti-inflammatory, anti-oxidative, anti-epileptic, anti-psychotic, and neuroprotective

properties [22–24]. CBD is one of the most abundant and therapeutically relevant phytocannabinoids in the *Cannabis* plant, devoid of the psychoactive side effect [25]. So far, molecular targets involved in various therapeutic properties produced by CBD are not fully understood. Furthermore, few studies were performed in order to examine the effects of CBD with respect to obesity and its complications. Thus, the aim of the present study was to investigate whether chronic CBD administration affects the content of bioactive lipid species (e.g., CER or sphingosine-1-phosphate (S1P)), as well as insulin signal transduction, in red skeletal muscle (musculus gastrocnemius) of rats subjected to a high-fat diet (HFD) (Scheme 1). In our experimental model, we focused on the red skeletal muscle (consisting mainly of slow-twitch fibers) due to its predominant aerobic metabolism, in which a primary source of energy is based on the oxidation of glucose and FAs, as well as indicated insulin resistance [26].

2. Materials and Methods

2.1. Animals and Study Design

Male Wistar rats (70–100 g) were purchased from the Center for Experimental Medicine of the Medical University of Bialystok, Poland. The animals were kept under controlled conditions (22 °C ± 2, 12-h/12-h light/dark cycle) with unlimited access to tap water and standard rodent chow (Labofeed B, Animal Feed Manufacturer "Morawski", Kcynia, Poland). The study was approved by the Animal Ethics Committee in Olsztyn (No. 71/2018).

The animals were randomly assigned to four experimental groups after a period of acclimatization (seven days): (1) control group—rats fed a standard diet (kcal distribution: 12.4% of energy from fat, 57.1% from carbohydrates, and 30.5% protein), (2) CBD group—rats fed a standard diet and CBD-treated, (3) HFD group—rats fed a high-fat diet (kcal distribution: 60% of energy from fat, 20% from carbohydrates, and 20% protein), and (4) HFD + CBD group—rats fed a high-fat diet and CBD-treated. The total time course of feeding rats either standard chow or a high-fat diet lasted seven weeks, and each experimental group consisted of 10 rats. Starting from the fifth week, simultaneously with the respective diet, the rats received injections of CBD or its vehicle for the next two weeks of the experiment. Rats fed both a standard diet and an HFD were injected intraperitoneally (i.p.) with synthetic CBD (purity: ≥99%; THC Pharm GmbH, Frankfurt, Germany) in a dose of 10 mg/kg of body mass (3:1:16, ethanol, Tween-80, and 0.9% NaCl), and corresponding control and HFD groups received the vehicle once a day consecutively for 14 days. Twenty-four hours after the last dose of CBD or its solvent, rats from control groups, as well as HFD-fed groups, were anaesthetized by intraperitoneal injection of pentobarbital (80 mg/kg body mass). Muscle samples (red musculus gastrocnemius with oxidative metabolism) were collected, and visible fatty tissue was mechanically removed. Subsequently, the samples were immediately frozen using aluminum tongs precooled in liquid nitrogen and stored at −80 °C until further analyses. Blood samples were obtained through inferior vena cava puncture and collected into heparinized tubes and centrifuged; then, plasma was separated.

2.2. Plasma Measurements

Plasma glucose and insulin concentrations were measured using a Glucose Colorimetric Assay Kit II (BioVision Inc., Milpitas, CA, USA) and Rat Insulin ELISA Kit (Mercodia AB, Uppsala, Sweden), respectively, following the manufacturer's instructions. The intensity of colored product was measured in a hybrid multi-mode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA) and, for each measurement, calculated values were based on a separate standard curve. Additionally, the insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), where fasting plasma glucose (FPG) concentration was expressed in millimoles per liter and fasting plasma insulin was expressed in microunits per milliliter (HOMA-IR = (FPG × FPI)/22.5).

2.3. Intramuscular Glycogen Analysis

The intramuscular glycogen content was determined using a colorimetric method (Glycogen Colorimetric Assay Kit II, BioVision Inc., Milpitas, CA, USA) according to the manufacturer's protocol.

Briefly, skeletal muscle samples were homogenized in double-distilled water; subsequently, tissues were boiled in order to inactivate enzymes and then centrifuged. Appropriate reagents were added to the collected supernatants and, after 30 minutes of incubation at room temperature. the absorbance of glycogen products was measured in a hybrid multi-mode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA). Calculated values were based on a standard curve, and glycogen concentration was expressed in micrograms per microliter.

2.4. Skeletal Muscle Lipid Analysis

The contents of ceramide (CER), sphinganine (SFA), sphingosine (SFO), sphinganine-1-phosphate (SFA1P), and sphingosine-1-phosphate (S1P) in the skeletal muscle samples were measured by the means of high-performance liquid chromatography (HPLC), as previously reported [27]. In brief, tissues were homogenized, and lipids were extracted by the addition of chloroform. The lipid extracts were transferred to a fresh tube with pre-added 40 pmol of *N*-palmitoyl-D-erythro-sphingosine (C17 base) as an internal standard. Afterward, the samples were washed with alkaline water to form deacylate ceramide. The obtained lipid residues released from ceramide were converted to their *o*-phthalaldehyde derivatives and analyzed using the HPLC system (PROSTAR; Varian Inc. (Palo Alto, CA, USA)) equipped with a fluorescence detector and C18 reversed-phase column (Varian Inc. OmniSpher 5, 4.6 × 150 mm).

2.5. Western Blotting

The total expression of proteins directly involved in sphingolipid and glucose metabolism, as well as components of the endocannabinoidome, was detected using a routine Western blotting procedure, as previously described [28]. Briefly, samples of the red skeletal muscle were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Then, the bicinchoninic acid method (BCA), with bovine serum albumin (BSA) as a standard, was used to ascertain protein concentration in the homogenates. After that, homogenates were diluted with Laemmli buffer, and the same amounts of protein (30 µg) were loaded onto CriterionTM TGX Stain-Free Precast Gels (Bio-Rad, Hercules, CA, USA). Subsequently, muscle homogenates were separated during electrophoresis and transferred onto nitrocellulose membranes. After blocking in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat dry milk or BSA, the membranes were incubated overnight with selected primary antibodies: insulin receptor substrate 1 (IRS-1, 1:1000; Cell Signaling Technology, Danvers, MA, USA), phosphorylated insulin receptor substrate 1 (pIRS1 (Ser302), 1:1000; Cell Signaling), protein kinase B (Akt/PKB, 1:1000; Cell Signaling Technology), phosphorylated protein kinase B (pAkt/PKB (Ser473), 1:1000; Cell Signaling Technology), AS160 protein (AS160, 1:500; Cell Signaling Technology) phosphorylated AS160 protein (pAS160, 1:500; Cell Signaling Technology), glycogen synthase kinase 3 (GSK3, 1:500; Thermo Scientific, Rockford, IL, USA), phosphorylated glycogen synthase kinase 3 (pGSK3 (Ser9), 1:500; Thermo Scientific), glucose transporter 1 (GLUT1, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), glucose transporter 4 (GLUT4, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), pyruvate dehydrogenase (PDH, 1:5000; Abcam, Cambridge, UK), serine palmitoyltransferase, long chain base subunit 1 (SPTLC1, 1:500; Abcam), ceramide synthase 5 (LASS5, 1:500; Thermo Scientific), acid ceramidase (ASAH1, 1:500; Santa Cruz Biotechnology), sphingosine kinase 2 (SPHK2, 1:500; Sigma Aldrich, Saint Louis, MO, USA), cannabinoid receptor 1 (CB1, 1:500; Abcam), cannabinoid receptor 2 (CB2, 1:500; Abcam), transient receptor potential channel 1 (TRPV1, 1:500; Santa Cruz Biotechnology), and serotonin receptor (5-HT1A, 1:3000; Thermo Scientific). Next, nitrocellulose membranes were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology). Thereafter, the protein bands were visualized using the appropriate substrate (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA), and obtained signals were quantified densitometrically with a ChemiDoc visualization system (Image Laboratory Software Version 6.0.1; Bio-Rad, Warsaw, Poland). The expression of selected target proteins was

quantified using stain-free gels and the total protein normalization method (Bio-Rad). All data are expressed as the percentage of the control group based on six independent determinations.

2.6. Statistical Analysis

All results are expressed as mean values ± SD. The data were subjected to the Shapiro–Wilk test and Bartlett's test to assess the distribution of values and homogeneity of the variance. Statistical differences between groups were determined based on the results of one-way ANOVA followed by an appropriate post hoc test using GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA, USA). Results were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effect of Chronic CBD Administration on Plasma Glucose and Insulin Concentrations, as Well as HOMA-IR, in Rats Subjected to Standard and High-Fat Diets

Our study demonstrated a pronounced decrease in plasma glucose level in the CBD group (−11.9%, $p < 0.05$; Table 1) compared to the control rats. Moreover, we noticed that both HFD-fed groups (untreated and treated with CBD) exhibited a significantly increased concentration of insulin (+89.9% and +45.2%, $p < 0.05$; Table 1, respectively) and considerably increased HOMA-IR index (+59.9% and +39.0%, $p < 0.05$; Table 1, respectively) in comparison with the control group. Importantly, we observed that two-week CBD treatment caused a substantial reduction in insulin concentration in the HFD group (−23.5%, $p < 0.05$; Table 1 vs. HFD group). Even though, chronic CBD administration decreased the HOMA-IR value in the HFD group compared to the corresponding untreated HFD group, the difference did not reach a significant level (−13.0%, $p > 0.05$; Table 1).

Table 1. Plasma glucose and insulin levels, as well as homeostatic model assessment for insulin resistance (HOMA-IR), after chronic cannabidiol (CBD) administration in rats fed standard (control group) and high-fat diets (HFD). The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ significant difference: control group vs. examined group; [b] $p < 0.05$ significant difference: HFD vs. HFD + CBD.

	Control	CBD	HFD	HFD + CBD
Glucose (mg/dL)	105 ± 8	93 ± 7 [a]	94 ± 7	100 ± 6
Insulin (μg/mL)	0.65 ± 0.12	0.70 ± 0.18	1.24 ± 0.19 [a]	0.95 ± 0.25 [a,b]
HOMA-IR	3.84 ± 0.25	3.57 ± 0.77	6.46 ± 1.40 [a]	5.62 ± 1.56 [a]

3.2. Effect of Chronic CBD Administration on the Sphingolipid Pathway (Sphinganine, Sphinganine-1-Phosphate, Ceramide, Sphingosine, and Sphingosine-1-Phosphate) in Skeletal Muscle of Rats Subjected to Standard and High-Fat Diets

In the experimental model of HFD-induced obesity, we observed a significant intensification of the de novo ceramide synthesis pathway, which resulted in an elevation of intramuscular content of SFA (+21.2%, $p < 0.05$; Figure 1A), SFA1P (+231.1%, $p < 0.05$; Figure 1B), CER (+25.7%, $p < 0.05$; Figure 1C), and SFO (+14.8%, $p < 0.05$; Figure 1D) after the HFD course in comparison with the control group. As expected, chronic CBD administration to rats fed the high-fat diet substantially reduced the content of the above-mentioned components of the sphingolipid pathway, i.e., SFA (–72.9%, $p < 0.05$; Figure 1A), CER (−14.9%, $p < 0.05$; Figure 1C), and SFO (−24.3%, $p < 0.05$; Figure 1D), in the red gastrocnemius muscle compared to the HFD group alone. The only component of the sphingolipid pathway which was enhanced by two-week CBD treatment in rats fed either the standard chow or the high-fat diet in the red gastrocnemius muscle was SFA1P (+306.7% and +325.0%, $p < 0.05$; Figure 1B vs. control group, respectively). Concomitantly, compared to the control conditions, rats from the CBD group exhibited significantly reduced content of both SFA and SFO (−53.6% and −26.3%, $p < 0.05$; Figure 1A,D, respectively) with no change in CER and S1P levels ($p > 0.05$; Figure 1C,E, respectively). Interestingly, the intramuscular content of S1P was decreased in the lipid overload condition (−21.8%,

$p < 0.05$; Figure 1E vs. control group) and subsequently elevated by CBD introduction in the same HFD group (+22.4%, $p < 0.05$; Figure 1E vs. HFD group). Similarly, the value of S1P/CER ratio was restored after CBD application in the high-fat diet group (−27.3%, $p < 0.05$; Figure 1F, HFD group vs. control group; +20.9%, $p < 0.05$; Figure 1F, HFD group vs. HFD + CBD group).

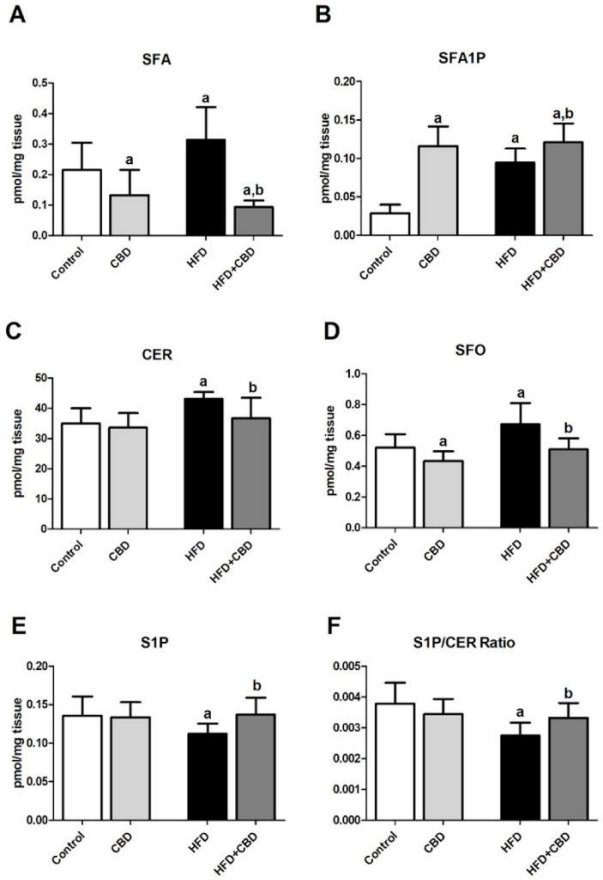


Figure 1. Intramuscular content of different components of sphingolipid pathway in rats after chronic cannabidiol (CBD) treatment, i.e., (**A**) sphinganine (SFA), (**B**) sphinganine-1-phosphate (SFA1P), (**C**) ceramide (CER), (**D**) sphingosine (SFO), (**E**) sphingosine-1-phosphate (S1P), and (**F**) sphingosine-1-phosphate/ceramide ratio (S1P/CER Ratio) in the red gastrocnemius muscle of rats fed a standard diet (control group) or high-fat diet (HFD). The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ significant difference: control group vs. examined group; [b] $p < 0.05$ significant difference: HFD vs. HFD + CBD.

3.3. Effect of Long-Term CBD Administration on the Total Intramuscular Expression of Proteins Involved in the Sphingolipid Metabolism in Rats Fed Standard and High-Fat Diets

Induction of obesity by high-fat diet feeding resulted in a significant increase in the total expression of SPTLC1 (+25.9%, $p < 0.05$; Figure 2A) in the red gastrocnemius muscle of the HFD group compared to the rats fed a standard chow, which was further declined by two-week CBD injections (−18.2%, $p < 0.05$; Figure 2A vs. HFD group). Similar effects compared to control conditions were observed in the high-fat diet group in regard to the total expression of LASS5 (+41.2%, $p < 0.05$; Figure 2B). Most importantly, the total intramuscular expression of this enzyme was considerably reduced in the chronic presence of CBD during the course of high fat feeding (−66.4%, $p < 0.05$; Figure 2B vs. control group and −76.2%, $p < 0.05$; Figure 2B vs. HFD group). Concomitantly, we did not notice any significant alternations in the total expression of ASAH1 and SPHK2 ($p > 0.05$; Figure 2C,D,

respectively) in the red gastrocnemius muscle of rats subjected to an HFD compared to the control subjects. Interestingly, two-week CBD treatment had a more pronounced effect on the ASAH1 (+54.6%, $p < 0.05$; Figure 2C vs. HFD group) and SPHK2 expression (+28.7%, $p < 0.05$; Figure 2D vs. HFD group) in the red skeletal muscle during high-fat feeding. Moreover, during feeding rats with standard chow, CBD treatment had just the opposite effect of lowering ASAH1 expression (−47.6%, $p < 0.05$; Figure 2C) compared to the control group.

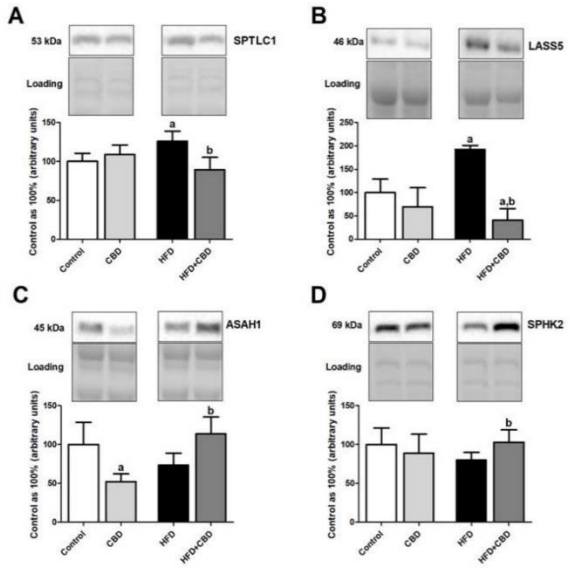


Figure 2. The total expression of proteins involved in the sphingolipid metabolism, e.g., (**A**) serine palmitoyltransferase, long chain base subunit 1 (SPTLC1), (**B**) ceramide synthase 5 (LASS5), (**C**) acid ceramidase (ASAH1), and (**D**) sphingosine kinase 2 (SPHK2), in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after chronic cannabidiol (CBD) treatment. The total expressions of the above proteins are presented as a percentage difference compared to the control group, which was set as 100%. The data are expressed as mean values ± SD, $n = 6$ in each group. [a] $p < 0.05$ significant difference: control group vs. examined group; [b] $p < 0.05$ significant difference: HFD vs. HFD + CBD.

3.4. Effect of Chronic CBD Administration on the Total Expression and Phosphorylation of Insulin Pathway Proteins, Glucose Transporters, and Glycogen Content in Skeletal Muscle of Rats Fed Standard and High-Fat Diets

In the skeletal muscle, we observed that rats fed an HFD showed a substantial decrease in the phosphorylation of proteins involved in insulin signaling pathway, i.e., IRS-1 (−13.8%, $p < 0.05$; Figure 3A, Figure S1A) and GSK-3 (−24.1%, $p < 0.05$; Figure 3D, Figure S1) in comparison with the control rats fed a standard diet. Concomitantly, we noticed a substantial elevation in the total muscular expression of GLUT1 and GLUT4 (+62.9% and +56.4%, $p < 0.05$; Figure 3E,F, respectively) with a parallel decrease in the content of glycogen in the red gastrocnemius muscle (−43.3%, $p < 0.05$; Figure 3H) in the HFD group compared to the control group. On the other hand, chronic CBD treatment resulted in a significant restoration in intramuscular phosphorylation of Akt (Ser-473) (+59.5%, $p < 0.05$; Figure 3B vs. HFD group) and GSK-3 (+38.4%, $p < 0.05$; Figure 3D vs. HFD group) compared to HFD alone. Concomitantly, CBD administration to animals being on an HFD resulted in a pronounced reduction of the total expression of both GLUT1 and GLUT4 (−32.9% and −30.8%, $p < 0.05$; Figure 3E,F, respectively) together with restoration of intramuscular glycogen pool (+56.4%, $p < 0.05$; Figure 3H) in comparison with HFD group. The above-mentioned effects of prolonged CBD treatment in high-fat diet rats were completed by markedly elevated total PDH expression (+37.9%, $p < 0.05$; Figure 3G vs. HFD group).

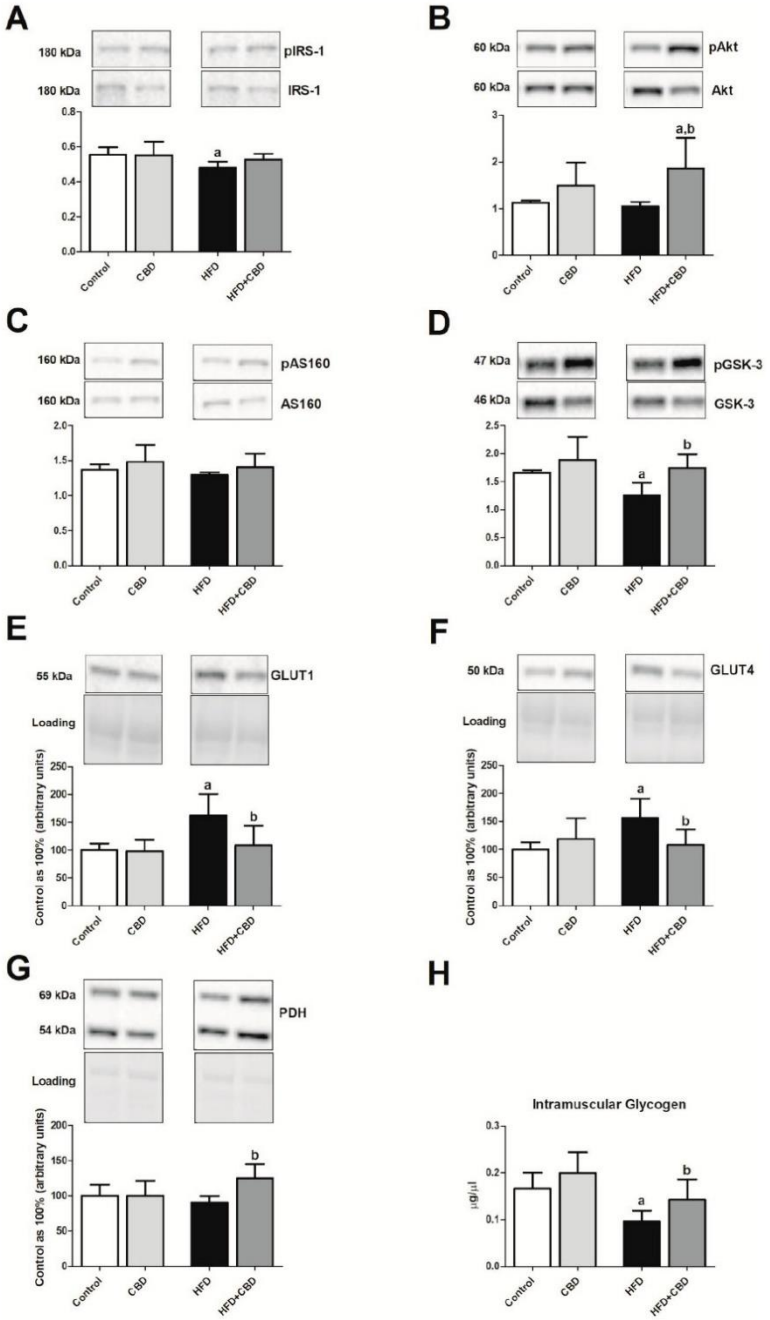


Figure 3. The ratio of total expression of phosphorylated and unphosphorylated proteins involved in insulin signaling pathway, e.g., (**A**) phosphorylated insulin receptor substrate 1/insulin receptor substrate 1 (pIRS-1/IRS-1), (**B**) phosphorylated protein kinase B/protein kinase B (pAkt/Akt), (**C**) phosphorylated AS160 protein/AS160 protein (pAS160/AS160), and (**D**) phosphorylated glycogen synthase kinase 3/glycogen synthase kinase 3 (pGSK-3/GSK-3), as well as total expression of (**E**) glucose transporter 1 (GLUT1), (**F**) glucose transporter 4 (GLUT4), (**G**) pyruvate dehydrogenase (PDH), and (**H**) glycogen content in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after chronic cannabidiol (CBD) treatment. The total expressions of the above proteins are presented as a percentage difference compared to the control group, which was set as 100%. The data are expressed as mean values ± SD, $n = 6$ in each group. [a] $p < 0.05$ significant difference: control group vs. examined group; [b] $p < 0.05$ significant difference: HFD vs. HFD + CBD.

3.5. Effect of Chronic CBD Administration on the Total Intramuscular Protein Expression of Endocannabinoid System Components in Rats Subjected to Standard and High-Fat Diets

Our experiment demonstrated that the HFD group presented significantly elevated total expression of eCBome receptors, i.e., CB_1 (+47.9%, $p < 0.05$; Figure 4A), TRPV1 (+61.9%, $p < 0.05$; Figure 4C), and 5-HT1A (+93.3%, $p < 0.05$; Figure 4D) in comparison with the control group fed standard chow. Unexpectedly, the total intramuscular expressions of the above-mentioned receptors in the lipid overload conditions and CBD presence were considerably decreased (CB_1: −33.9%, $p < 0.05$; Figure 4A; TRPV1: −35.4%, $p < 0.05$; Figure 4B; 5-HT1A: −62.2%, $p < 0.05$; Figure 4D) compared to the rats subjected only to an HFD. Concomitantly, we did observe an increase in the total muscular expression of CB_2 only in the case of animals fed a standard and high-fat chow and being injected with CBD (+40.6%, $p < 0.05$; Figure 4B vs. control group and +43.5%, $p < 0.05$; Figure 4B vs. HFD group).

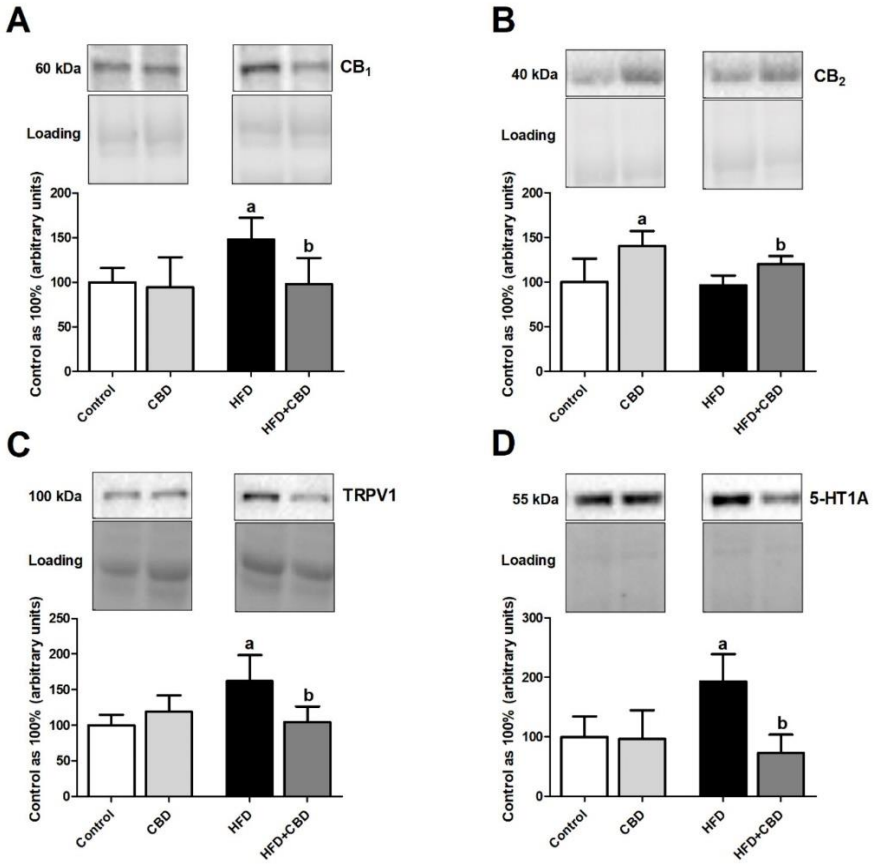


Figure 4. The total expression of (**A**) cannabinoid receptor 1 (CB_1), (**B**) cannabinoid receptor 2 (CB_2), (**C**) transient receptor potential channel 1 (TRPV1), and (**D**) serotonin receptor (5-HT1A) in red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after chronic cannabidiol (CBD) treatment. The total expressions of the above proteins are presented as a percentage difference compared to the control group, which was set as 100%. The data are expressed as mean values ± SD, $n = 6$ in each group. a $p < 0.05$ significant difference: control group vs. examined group; b $p < 0.05$ significant difference: HFD vs. HFD + CBD.

4. Discussion

CBD, a non-psychotropic constituent of marijuana, exerts potentially beneficial pharmacological effects for obesity treatment. Therefore, according to our knowledge, for the first time, we examined, in a rat model of HFD-induced obesity and related whole-body insulin resistance, the impact of CBD

on sphingolipids and glucose metabolism in the red skeletal muscle. It is important to note that the present study revealed the link between ceramide and other sphingolipid derivatives, ECS, and insulin signal transduction.

As we mentioned, one of our aims was to determine the effect of CBD on the intramuscular content of selected sphingolipids (e.g., SFA, CER, S1P) in a model of HFD-induced obesity. During obesity, adipocytes are overloaded, which results in the accumulation of bioactive lipids in excessive amounts in several tissues, such as skeletal and cardiac muscle [29]. This phenomenon is known as lipotoxicity and contributes to the development of insulin resistance; however, the exact mechanism is not yet well characterized [30,31]. Accumulation of lipid intermediates, including CER, can directly interfere with the insulin signaling pathway [29]. In particular, ceramides impair insulin signal transduction by activating protein kinase C λ/ζ (PKC λ/ζ), implicated in the dephosphorylation and reduction in protein kinase B (Akt/PKB) activity, as well as stimulation of IκB kinase (IKK) and c-Jun N-terminal kinase (JNK), which attenuates insulin receptor substrate 1 (IRS-1) phosphorylation [32,33]. Importantly, our results demonstrated that CBD can be effective in ameliorating lipotoxicity and related insulin resistance due to observed alternations in the content of several sphingolipids. The current experiment showed markedly decreased SFA content after CBD treatment, thereby demonstrating a decline in the first step of the de novo pathway of ceramide synthesis, which was shown to be intensified during obesity [26]. Additionally, the above changes are in line with a decrease in SPTLC1 and LASS5 expressions (an enzymes involved in the de novo ceramide synthesis, Scheme 1) after CBD administration in rats fed an HFD [32]. This favorable effect of CBD action was manifested primarily by pronounced reduction in the intramyocellular CER content. Furthermore, ceramide derivatives, such as SFO and S1P, can also influence cellular survival, growth, and various functions and, thus, they may be involved in metabolic disorders [34]. SFO is reported as a proapoptotic molecule, whereas conflicting reports regarding S1P function in skeletal muscle can be found [35,36]. Several studies indicate that an increase in the S1P formation contributes to the IR [37]. However, a growing body of evidence described that S1P has just the opposite effects to CER and promotes cell proliferation and survival [38]. The current study showed a significant increase in intramyocellular SFO content in rats fed an HFD, whereas CBD substantially reduced its amount in favor of enhancing S1P content. This, in turn, resulted in an elevation of S1P/CER ratio and emphasized an improvement in sphingolipid rheostat imbalance due to IR [38]. The lower value of S1P/CER ratio observed in the HFD group may be associated with impaired insulin signal transduction, as well as enhanced cellular apoptotic processes. Furthermore, we observed changes in the total intramuscular expression of enzymes involved in the conversion of CER to its derivatives. Chronic CBD treatment of rats subjected to the fatty acid oversupply caused a considerable elevation in the total ASAH1 expression (conversion of ceramide to sphingosine; Scheme 1) and simultaneously reduced its expression in the standard diet group. These data confirmed that CBD also diminished accumulation of proapoptotic sphingolipids in rats fed a standard diet. Nevertheless, after CBD administration, we noticed an increase in the total SPHK2 expression, which is a kinase responsible for the maintenance of a balance between proapoptotic and proliferative precursors [38]. Moreover, Bruce et al. [39] demonstrated that SPHK1 overexpression prevents intramuscular ceramide accumulation by promoting its conversion into S1P and, thus, attenuates insulin resistance. Therefore, targeting enzymes involved in the maintaining an equilibrium between CER and S1P levels may be a beneficial strategy for improving muscle insulin sensitivity. Hence, it should be underlined that, in our research, we provide evidence for promising effects of CBD in regard to sphingolipid metabolism in the condition of lipid oversupply.

Moreover, our data showed that feeding rats an HFD attenuated whole-body insulin sensitivity since, after seven weeks of the experiment, we observed an increase in the concentration of insulin content. The occurrence of IR after a high-fat feeding was confirmed by the elevation in the HOMA-IR value, which is consistent with other researchers' results [40–42]. In a number of studies, it was shown that increased fatty acid supply in a diet directly interferes with intracellular insulin signal pathways, leading to disturbances in whole-body glucose metabolism, together with a reduction

of glycogen synthesis in skeletal muscle, which is again in line with the results obtained in our research [30,43,44]. Even though the data presented herein indicated that the two-week time frame of CBD treatment is too short in order to substantially improve whole-body IR, it was revealed that CBD ameliorates the deteriorated intramuscular insulin pathway in rats subjected to HFD, mainly by enhancing the phosphorylation ratio of proteins involved in the downstream signaling of that hormone (i.e., Akt and GSK-3). This is in line with the recent research conducted by Fellous et al., who showed that CBD treatment (5 μM) of bone marrow mesenchymal stem cells (BM-MSCs) prevented the palmitate-induced insulin resistance by increasing *Glut4* messenger RNA (mRNA) expression with simultaneous full restoration of Akt activation and subsequent glucose uptake [45]. In parallel, as the consequence of chronic CBD administration, we observed restored glycogen depletion in the red gastrocnemius, which resulted from increased phosphorylation of Akt and further GSK-3 inhibition. Furthermore, it is well established that a high-fat feeding results in impaired translocation of glucose transporter 4 from the intramyocellular compartments to the plasma membrane in insulin sensitive tissues [46,47]. In our study, we noticed considerably elevated total expression of both GLUT4 and GLUT1 in rats after a seven-week course of HFD, whereas CBD administration substantially reduced their skeletal muscle expression in the same group of examined animals. We hypothesize that the increase in the total expression of glucose transporters in HFD group was a compensatory effect, since parallel glycogen depletion was observed. Importantly, the aforementioned increase in GLUT4 and GLUT1 expressions was attenuated by chronic CBD treatment in the HFD group, most probably as the consequence of reduced plasma insulin concentration and an improvement in its downstream signaling (increased pAkt/Akt and pGSK-3/GSK-3 ratios) in red gastrocnemius. Even though, we did not measure plasmalemmal expression of GLUT4 in our study, it seems that CBD through regulation of signaling proteins stimulated intramyocellular trafficking of the GLUT4 transporter, which resulted in restoration of intramuscular glycogen and elevated expression of oxidative enzymes (i.e., increased expression of PDH). Moreover, we did observe alternations in the value of phosphorylated to unphosphorylated signaling proteins ratios only in the case of pAkt/Akt and pGSK-3/GSK-3, presumably due to lack of a direct stimulation of isolated skeletal muscle strips by insulin in ex vivo conditions, which may be considered as a limitation of the study.

Previous studies showed that ECS is overactivated during obesity and, thus, it became a potential target of therapeutic interventions [17,20]. In order to determine the effect of CBD on the ECS in skeletal muscle of rats fed a standard chow and HFD, we examined the total expression of cannabinoid (CB_1 and CB_2) and non-cannabinoid receptors (TRPV1 and 5-HT1A). Our experiment showed that high-fat feeding resulted in a substantial elevation of the total CB_1 expression in a rat's skeletal muscle. These results are consistent with findings of other researchers and may be associated with increased levels of endocannabinoids during obesity, especially AEA, which is a partial agonist of the CB_1 receptor [17,18]. Furthermore, evidence was recently provided that CB_1 activation induced by an HFD suppresses the insulin-dependent phosphorylation of Akt through IRS-1 phosphorylation at Ser-307, thereby mediating the emergence of insulin resistance [48]. Furthermore, Trillou et al. demonstrated that $CB_1^{-/-}$ mice are resistant to HFD-induced obesity [49]. Importantly, we noted that CBD treatment significantly decreased the expression of CB_1 in the red gastrocnemius of rats subjected to an HFD. Our data are also in line with those obtained by Laprairie et al., since they demonstrated that CBD is a negative allosteric modulator of the CB_1 receptor [50]. On the other hand, in the case of the CBD effect on CB_2 receptors, several studies showed contradictory data, describing its activity as an agonist or inverse agonist of these receptors [51,52]. Our research reported that CBD significantly increased total CB_2 expression in both control and high-fat diet-fed animals. The aforementioned alternations in the expression of cannabinoid receptors in skeletal muscle of high-fat diet-fed rats are in agreement with previous findings describing a positive relationship between CB_1 receptors and oxidative stress [53] and an opposite effect in the case of CB_2 receptors [53]. This should be underlined owing to the fact that obesity and related metabolic disturbances coincide with the promotion of oxidative stress. On the contrary, recent evidence emerged that, due to the low affinity of CBD for CB_1 and CB_2 receptors,

it induces its effects primarily through other molecular targets, including TRPV1 channels and 5-HT1A receptors. The exact role of TRPV1 and 5-HT1A in obesity is not yet characterized, and additional research is needed to understand their molecular mechanism of action, as well as in the course of insulin resistance. In the current study, we showed elevated intramuscular TRPV1 and 5-HT1A expressions in HFD fed rats. We hypothesize that these changes may be associated with an increased level of endocannabinoids during obesity, in particular AEA, which is an agonist of those receptors [54]. Noteworthy, our research revealed that CBD administration to HFD-subjected animals resulted in a significant reduction in the expression of these receptors, indicating that CBD interferes with their activation. Such a conclusion arises since it is confirmed that CBD exhibits agonistic activity on the TRPV1 and 5-HT1A receptors [55,56]. Interestingly, recent research demonstrated that CBD, mostly via TRPV1 activation, enhanced murine C2C12 myoblast differentiation, together with inflammation reduction and autophagy restoration in in vivo conditions, which supported our notion concerning the protective role of CBD in the skeletal muscle [57]. Taken altogether, the data presented herein support the hypothesis that the ECS is involved in the development of metabolic disorders including insulin resistance. Moreover, this finding raises the possibility that CBD may be a useful tool in the treatment of obesity and its comorbidities by acting on the ECS, not only on receptors, but also on ligands and their metabolic routes.

5. Conclusions

In summary, our data provide new insight into the mechanism of cannabidiol action at the cellular level in skeletal muscle. We reported, for the first time, that chronic CBD treatment. on the one hand, prevented intramyocellular accumulation of CER and SFA, but, on the other hand, elevated S1P in FA oversupply in a diet. Moreover, we found that CBD improves downstream insulin signaling and the oxidative metabolism of glucose, while it restores glycogen depletion in myocytes during high-fat feeding. Furthermore, taking into consideration some limitations of the study, it seems that a two-week CBD treatment is too short to markedly diminish whole-body IR in obese subjects. Nevertheless, a two-week CBD treatment is enough to effectively inhibit the de novo ceramide synthesis pathway, thereby reducing lipotoxicity and provoking an insulin-sensitizing effect in the myocytes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/9/1241/s1: Figure S1. Representative Western blots showing the ratio of the total expression of phosphorylated and unphosphorylated proteins involved in the insulin signaling pathway.

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10. Article No. 3

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Attenuation of oxidative stress and inflammatory response by chronic cannabidiol administration is associated with improved n-6/n-3 PUFA ratio in the white and red skeletal muscle in a rat model of high-fat diet-induced obesity.

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Attenuation of Oxidative Stress and Inflammatory Response by Chronic Cannabidiol Administration Is Associated with Improved n-6/n-3 PUFA Ratio in the White and Red Skeletal Muscle in a Rat Model of High-Fat Diet-Induced Obesity

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Abstract: The consumption of fatty acids has increased drastically, exceeding the nutritional requirements of an individual and leading to numerous metabolic disorders. Recent data indicate a growing interest in using cannabidiol (CBD) as an agent with beneficial effects in the treatment of obesity. Therefore, our aim was to investigate the influence of chronic CBD administration on the n-6/n-3 polyunsaturated fatty acids (PUFAs) ratio in different lipid fractions, inflammatory pathway and oxidative stress parameters in the white and red gastrocnemius muscle. All the designed experiments were performed on Wistar rats fed a high-fat diet (HFD) or a standard rodent diet for seven weeks and subsequently injected with CBD (10 mg/kg once daily for two weeks) or its vehicle. Lipid content and oxidative stress parameters were assessed using gas–liquid chromatography (GLC), colorimetric and/or immunoenzymatic methods, respectively. The total expression of proteins of an inflammatory pathway was measured by Western blotting. Our results revealed that fatty acids (FAs) oversupply is associated with an increasing oxidative stress and inflammatory response, which results in an excessive accumulation of FAs, especially of n-6 PUFAs, in skeletal muscles. We showed that CBD significantly improved the n-6/n-3 PUFA ratio and shifted the equilibrium towards anti-inflammatory n-3 PUFAs, particularly in the red gastrocnemius muscle. Additionally, CBD prevented generation of lipid peroxidation products and attenuated inflammatory response in both types of skeletal muscle. In summary, the results mentioned above indicate that CBD presents potential therapeutic properties with respect to the treatment of obesity and related disturbances.

Keywords: cannabidiol; cannabis; inflammation; insulin resistance; lipids; oxidative stress

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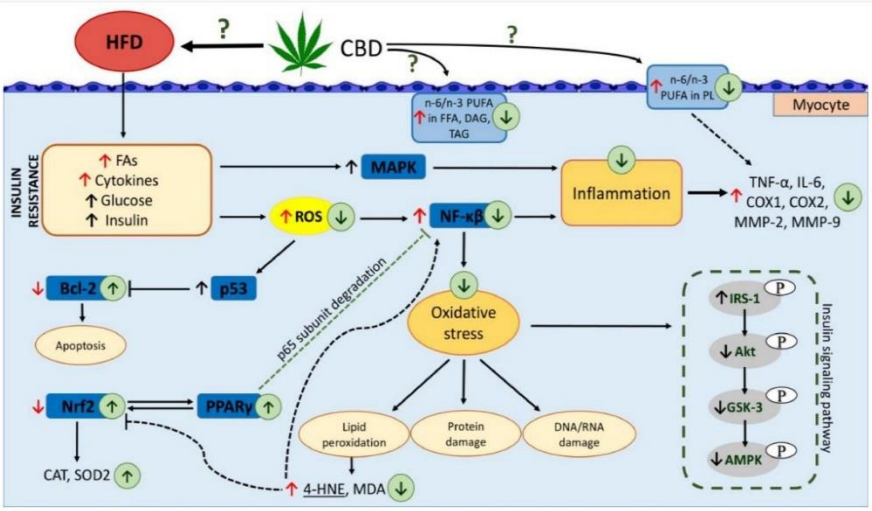
1. Introduction

Nowadays, according to the World Health Organization, obesity is one of the most significant health problems of the 21st century [1]. An increased prevalence of obesity in the world is attributed to many factors, including overnutrition, sedentary lifestyle, as well as many environmental and genetic factors, which were confirmed by a number of epidemiological and clinical studies [2]. During the progression of obesity, the excessive amounts of lipids are deposited in non-adipose tissues (e.g., liver, skeletal and cardiac muscle) leading to dyslipidemia, hyperglycemia and hyperinsulinemia [3–5]. These metabolic complications strongly correlate with the development of insulin resistance (IR), the occurrence of which plays a pivotal role in the pathogenesis of many chronic diseases such as type 2 diabetes mellitus (T2D), metabolic syndrome (MetS) and cardiovascular diseases (CVDs) [6,7].

During this past decade, several studies reported the relevant role of chronic inflammation in the development of IR [8]. It was widely demonstrated that chronic overnutrition

elicits an inflammatory response leading to systemic and tissue-specific low-grade inflammation through the release of proinflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), as well as production of reactive oxygen species (ROS), which directly attenuate insulin signaling in insulin-sensitive tissues (Scheme 1) [8–10]. What is more, a high-fat diet (HFD) provides large amounts of polyunsaturated fatty acids (PUFAs), which are substrates for the synthesis of signaling molecules, eicosanoids (e.g., prostaglandins, prostacyclins, leukotrienes and thromboxanes) that affect functions of many tissues and organs in physiological and pathological conditions [11].



Scheme 1. Effects of a high-fat diet (HFD) and two-week cannabidiol (CBD) administration on the n-6/n-3 polyunsaturated fatty acids (PUFAs) ratio in different lipid fractions (free fatty acids (FFAs), diacylglycerols (DAGs), triacylglycerols (TAGs) and phospholipids (PLs)), oxidative stress and inflammatory pathway in rat myocytes; ↑, increase; ↓, decrease; red arrow indicates the effects of seven weeks of high-fat diet feeding; black arrow indicates the effects of obesity; green arrow indicates the effects of two weeks of CBD treatment in high-fat diet fed rats; fatty acids (FAs); mitogen-activated protein kinase (MAPK); reactive oxygen species (ROS); p53 protein (p53); B cell lymphoma 2 (Bcl-2); nuclear factor κB (NF-κB); tumor necrosis factor α (TNF-α); interleukin 6 (IL-6); cyclooxygenase 1 (COX1); cyclooxygenase 2 (COX2); matrix metalloproteinase-2 (MMP-2), matrix metallo-proteinase-9 (MMP-9), peroxisome proliferator-activated receptor gamma (PPARγ); nuclear factor erythroid 2-related factor 2 (Nrf2); catalase (CAT), superoxide dismutase 2 (SOD2); 4-hydroxynonenal (4-HNE); malonyldialdehyde (MDA); insulin receptor substrate 1 (IRS-1), protein kinase B (Akt); glycogen synthase kinase 3 (GSK-3); 5′AMP-activated protein kinase (AMPK).

Moreover, data from numerous studies indicate that another mechanism which activates the proinflammatory cascade and appears to be of central importance is oxidative stress, which is defined as the imbalance between the production of ROS and reactive nitrogen species (RNS), and the effectiveness of enzymatic (e.g., catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)) and non-enzymatic (e.g., reduced glutathione (GSH)) antioxidant protection [12]. Redox balance alterations in favor of ROS/RNS overproduction cause peroxidation of proteins and lipids as well as oxidative damage of nucleic acids, which may result in damage to cellular structures, including membranes, mitochondria and DNA (Scheme 1) [13].

In recent years, numerous studies based on newly emerging data have confirmed that the endocannabinoidome (eCBome) is a key player in the regulation of energy metabolism and its alterations. This complex lipid signaling system is also involved in the control of thermogenesis, neuromodulatory action and inflammatory processes [14,15]. By the turn of the last century, it was established that eCBome consists of endogenous ligands derived from

long-chain PUFAs known as endocannabinoids (ECs), mostly *N*-arachidonoylethanolamine (anandamide (AEA)) and 2-arachidonoylglycerol (2-AG), with their own anabolic and catabolic pathways [16]. The action of ECs is mediated via widespread cannabinoid receptors CB_1 and CB_2, as well as many other molecular targets [17–19]. It is important to remark that ECs themselves have many structural congeners, and these compounds exist in dynamic balance with other lipid-derived mediators, including prostamides and eicosanoids [15]. Additionally, ECs serve as an endogenous source of arachidonic acid (AA) which is generated from AEA and 2-AG via the fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively [20].

Over the past century, the *Cannabis sativa* plant has been extensively studied for its medical properties. So far, more than 120 terpenophenolic compounds have been isolated from this medicinal plant [21]. Among all phytocannabinoids, cannabidiol (CBD) has been in the spotlight for several decades due to its excellent safety profile, lack of psychoactive effects and plenty of indicated therapeutic properties including neuroprotective, analgesic, anti-epileptic, anti-oxidative, anti-inflammatory and potential anti-obesity properties [22–24]. Considering "classic" cannabinoid CB_1 and CB_2 receptors, CBD has a very low affinity for these receptors, whereas it has been reported that this phytocannabinoid can modulate diverse G protein-coupled receptors (GPCRs) (e.g., GPR55 and GPR18), thermosensitive transient receptor potential (TRP) channels (vanilloid type-1 receptor, TRPV1) as well as opioid and peroxisome proliferator-activated receptors (PPARs: PPARα and PPARγ) [23,25,26]. Additionally, CBD has also been shown to alter the eCBome tone by inhibiting FAAH and subsequent AEA hydrolysis [27]. All the above mechanisms of CBD action demonstrate how complex the pharmacology it exhibits is, which must be taken into consideration in order to understand its therapeutic potential under various pathophysiological conditions. Taking this into consideration, the present study aimed to investigate the impact of CBD on the n-6/n-3 PUFA ratio in different lipid fractions (free fatty acids (FFAs), diacylglycerols (DAGs), triacylglycerols (TAGs) and phospholipids (PLs)), oxidative stress parameters as well as the inflammatory pathway in the white and red skeletal muscle (musculus gastrocnemius) of rats with obesity induced by a high-fat diet. Moreover, our research reveals a comprehensive relationship between the endocannabinoid system, the influence of n-6, n-3 PUFA contents and the oxidative stress along with inflammation associated with obesity. Furthermore, the study compares the CBD's influence on two different metabolic types of skeletal muscle.

2. Materials and Methods

2.1. Animals and Experimental Protocol

The experiment was carried out on male Wistar rats weighing approximately 70–100 g obtained from the Center for Experimental Medicine of the Medical University of Bialystok, Poland. The animals were housed under standard holding conditions (22 ± 2 °C with a cycle of 12 h light/12 h dark) in plastic cages with unrestricted access to water and commercial pellet chow (Labofeed B, animal feed manufacturer "Morawski," Kcynia, Poland). All the conducted procedures were evaluated and approved by the animal ethics committee in Olsztyn (No. 71/2018). The rats, after a period of acclimatization (seven days), were randomly divided into four groups: (1) control group fed a standard rodent diet (containing 12.4 kcal% fat, 57.1 kcal% carbohydrates and 30.5 kcal% protein), (2) CBD group fed a standard rodent diet and administered CBD, (3) HFD group fed a high-fat diet (containing 60 kcal% fat, 20 kcal% carbohydrates and 20 kcal% protein) and (4) HFD + CBD group fed a high-fat diet and administered CBD. Each experimental group consisted of ten rats. For seven weeks of the study, the animals received a standard diet or an HFD, and starting from the sixth week, rats were injected intraperitoneally (i.p.) with CBD or its vehicle. Respective control and HFD fed rats were injected i.p. once a day for two weeks with synthetic CBD (10 mg/kg, purity ≥ 99%; THC Pharm GmbH, Frankfurt, Germany) or its solvent (3:1:16, ethanol, Tween-80 and 0.9% NaCl). At the end of the experiment, twenty-four hours after the last dose of CBD or its vehicle, rats were anaesthetized i.p.

with pentobarbital (80 mg/kg of body weight). Thereafter, whole blood was collected into test tubes with heparin through an inferior vena cava puncture and centrifuged to separate plasma. Muscle samples (red gastrocnemius muscle with predominant oxidative metabolism and white gastrocnemius muscle with largely anaerobic metabolism) were excised, and visible fatty tissue was mechanically removed. The obtained samples were at once frozen with aluminum tongs precooled in liquid nitrogen and then stored at −80 °C until final examination. Throughout the whole experiment, body weight of each rat was monitored (we observed significantly increased body weight in HFD fed rats; however, chronic CBD administration did not substantially affect the body weight in rats fed either a standard chow or a high-fat diet). Moreover, at the end of the experiment, we evaluated glucose and insulin concentrations as well as the HOMA-IR value, which was published previously [28].

2.2. Analysis of the Muscle and Plasma Lipid Contents

Intramuscular (FFA, DAG, TAG and PL) and plasma lipid (FFA and TAG) contents were determined by means of gas–liquid chromatography (GLC) as previously described [29]. In brief, the frozen muscle samples were pulverized with aluminum mortar precooled in liquid nitrogen. Subsequently, the muscle tissue and plasma lipids were extracted in a chloroform–methanol (2:1 vol/vol) solution using the Folch method [30]. Then, FFA, DAG, TAG and PL fractions were separated by thin-layer chromatography (TLC) on silica gel plates (Silica Plate 60, 0.25 mm; Merck, Darmstadt, Germany). Thereafter, the individual fatty acid methyl esters were quantified according to the standard retention times using GLC (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column). Total intramuscular FFA, DAG, TAG and PL as well as plasma FFA and TAG concentrations were estimated as the sum of the particular fatty acid species content in the selected fraction and expressed in nanomoles per gram of wet tissue and as nanomoles per milliliter in blood plasma.

2.3. Determination of Oxidative and Antioxidative Parameters

In order to determine the oxidative stress parameters, samples of the white and red skeletal muscles were homogenized in an ice-cold phosphate-buffered saline (PBS) with the addition of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Manheim, Germany) at 20 mg of tissue/1 mL PBS for catalase (CAT), superoxide dismutase 2 (SOD2) and total antioxidant capacity (TAC); at 10 mg of tissue/90 µL PBS for advanced glycation end product (AGE) and 4-hydroxynonenal (4-HNE); in a radioimmunoprecipitation assay (RIPA) buffer containing a cocktail of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Manheim, Germany) at 25 mg of tissue/250 µL RIPA for malondialdehyde (MDA) determination. Subsequently, the homogenates in the PBS were centrifuged for five minutes at 12,000× *g* at 4 °C to assess CAT, SOD2 and TAC, and for evaluation of AGE and 4-HNE, they were centrifuged at 5000× *g* for five minutes at 4 °C. For MDA determination, homogenates in the RIPA buffer were centrifuged for 10 min at 1600× *g* (4 °C). Thereafter, the supernatants were collected and stored in aliquots at −80 °C for later use.

The concentrations of CAT and SOD2 in muscle homogenates were measured with the use of a commercial ELISA kit from Cloud-Clone Corp. (Houston, TX, USA) following the manufacturer's instructions. The intensity of colored products was determined at the 450-nm wavelength in a hybrid multimode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA) and, for each measurement, the calculated values were based on the obtained standard curve. The CAT and SOD2 concentrations were expressed in nanograms and picograms per milligram of tissue, respectively.

In order to evaluate the TAC parameter in muscle samples, we used a colorimetric method (TAC assay kit, Abcam, Cambridge, UK). The absorbance was measured colorimetrically at 570 nm (Synergy H1TM, BioTek Instruments, Winooski, VT, USA). The value of the TAC parameter was calculated according to the manufacturer's instructions and expressed in nanomoles per milligram of tissue.

To determine intramuscular MDA content, we used a commercial kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA) using the thiobarbituric acid reactive substances (TBARS) method. The intensity of reaction products (MDA–TBA adducts) was measured colorimetrically at the 530-nm wavelength in a hybrid multimode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA). MDA concentration was calculated based on the standard curve and expressed as nanomoles per milligram of tissue.

Intramuscular AGE and 4-HNE concentrations were assessed using ELISA kits from Biorbyt (Cambridge, UK) according to the manufacturer's protocol. The absorbance was measured spectrophotometrically at the 450-nm wavelength in a hybrid multimode microplate reader (Synergy H1TM, BioTek Instruments, Winooski, VT, USA). The values of AGE and 4-HNE were calculated from standard curves and expressed in nanograms and picograms per milligram of tissue, respectively.

2.4. Western Blotting

Routine Western blotting procedure was used to determine total protein expression as it was reported in detail previously [31,32]. Briefly, red and white muscle samples were homogenized in an ice-cold RIPA buffer with the addition of protease and phosphatase inhibitors (Roche Diagnostics GmbH, Manheim, Germany). The determination of the total protein concentration in muscle homogenates was performed using the bicinchoninic acid (BCA) protein assay method with bovine serum albumin (BSA) as a standard. Subsequently, homogenates (30 μg of proteins) were reconstituted in the Laemmli buffer and separated on CriterionTM TGX Stain-Free precast gels (Bio-Rad, Hercules, CA, USA). Then, the proteins were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes in wet and semi-dry conditions, respectively. Next, the membranes were blocked in the Tris-buffered saline with Tween-20 (TBST) and 5% non-fat dry milk or 5% BSA and then incubated overnight at 4 °C with primary antibodies, i.e., cyclooxygenase-1 (COX-1, 1:500; Abcam, Cambridge, UK), cyclooxygenase-2 (COX-2, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 5-lipoxygenase (5-LO, 1:1500; Abcam, Cambridge, UK), 15-lipoxygenase (15-LO, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), peroxisome proliferator-activated receptor alpha (PPARγ, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), nuclear factor-kB (NF-kB, 1:500; Cell Signaling Technology Inc., Danvers, MA, USA), tumor necrosis factor α (TNF-α, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), interleukin 6 (IL-6, 1:3000; Abcam, Cambridge, UK), nuclear factor erythroid 2-related factor 2 (Nrf-2, 1:500; Abcam, Cambridge, UK), B cell lymphoma 2 (Bcl-2, 1:500; Cell Signaling Technology Inc., Danvers, MA, USA), matrix metalloproteinase-2 (MMP-2, 1:2500; Abcam, Cambridge, UK) and matrix metalloproteinase-9 (MMP-9, 1:5000; Abcam, Cambridge, UK). Thereafter, the membranes were incubated with horseradish peroxidase (HRP) and conjugated secondary antibodies (1:3000, Santa Cruz Inc., Dallas, TX, USA). In order to visualize protein bands, the chemiluminescence substrate (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA) was used, and then the obtained signals were quantified densitometrically with the use of the ChemiDoc visualization system (Image Laboratory Software Version 6.0.1; Bio-Rad, Warsaw, Poland). The expression of selected proteins was quantified with stain-free gels and the total protein normalization method (Bio-Rad, Hercules, CA, USA) (see Supplementary File S1). All the data are demonstrated as the percentage of the control group based on six independent determinations.

2.5. Statistical Analysis

The experimental data are expressed as mean values ± SD or percentage of the control group based on six independent determinations. The obtained results were subjected to the Shapiro–Wilk test and the Bartlett's test to assess the distribution of values and homogeneity of the variance. Statistical differences between groups were assessed by one-way test ANOVA followed by the appropriate post-hoc test with the use of GraphPad

Prism version 7.0 for Windows (GraphPad Software, La Jolla, CA, USA). The results were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effect of Two-Week CBD Treatment on the n-6 and n-3 PUFA Ratio in the White and Red Skeletal Muscles As Well As Plasma of Rats Subjected to Standard and High-Fat Diets

Induction of obesity by feeding rats a HFD resulted in a significant reduction in the pool of the n-6 PUFAs, i.e., of linoleic acid (LA) (−16.9%, $p < 0.05$), arachidonic acid (AA) (−10.1%, $p < 0.05$), and also of n-3 PUFAs, i.e., of α-linolenic acid (ALA) (−16.0%, $p < 0.05$) and eicosapentaenoic acid (EPA) (−17.5%, $p < 0.05$) in the FFA fraction (Table 1), in the white gastrocnemius muscle compared with the controls. We only observed an increase in the content of docosahexaenoic acid (DHA) (+32.9%, $p < 0.05$; Table 1) under the same conditions. Concomitantly, the rats fed both the standard and the HFD after the introduction of CBD exhibited a substantially elevated content of LA (+42.6% and +62.5%, $p < 0.05$, vs. control group, respectively; +95.6%, $p < 0.05$, vs. the HFD group; Table 1), AA (+52.8% and +173.7%, $p < 0.05$, vs. the control group, respectively; +204.5%, $p < 0.05$, vs. the HFD group; Table 1), ALA (+29.9%, $p < 0.05$, vs. the control group; +30.7%, $p < 0.05$, vs. the HFD group; Table 1) and DHA (+52.8% and +161.1%, $p < 0.05$, vs. the control group, respectively; +96.5%, $p < 0.05$, vs. the HFD group; Table 1) in the white skeletal muscle. However, compared to the control conditions, rats from the HFD group exhibited a considerably decreased EPA content after two-week CBD administration (−29.0%, $p < 0.05$; Table 1) in the same muscle type. In contrast, in the red gastrocnemius muscle, we noticed a significant increment in the fatty acid content of the FFA fraction, i.e., of LA (+76.0%), AA (+41.7%), ALA (+27.7%) and DHA (+75.1%) ($p < 0.05$; Table 2), in the rats subjected to a high-fat diet in comparison with the control rats, with the exception of EPA ($p > 0.05$; Table 2). Interestingly, the total intramuscular content of FFA fraction fatty acids in the rats fed either the standard or the high-fat diet was considerably greater in the chronic presence of CBD: LA (+39.2% and +63.5%, $p < 0.05$, vs. the control group, respectively; Table 1), AA (+41.7% and +99.3%, $p < 0.05$, vs. the control group, respectively; +40.6%, $p < 0.05$, vs. the HFD group; Table 2), ALA (33.7% and 22.0%, $p < 0.05$, vs. the control group, respectively; Table 2), EPA (+33.6% and +20.1%, $p < 0.05$, vs. the control group, respectively; +22.0%, $p < 0.05$, vs. the HFD group; Table 2) and DHA (+31.3% and +102.8%, $p < 0.05$, vs. the control group, respectively; +15.8%, $p < 0.05$, vs. the HFD group; Table 2) in the oxidative muscle.

Table 1. Polyunsaturated fatty acids (PUFA) content in the FFA fraction (nmol/g of wet tissue) in the white gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	57.59 ± 8.04	82.1 ± 14.10 [a]	49.51 ± 10.53 [a]	93.61 ± 7.54 [a,b]
(20:4n6)	17.47 ± 1.77	26.69 ± 6.90 [a]	15.70 ± 1.50 [a]	47.80 ± 5.31 [a,b]
(18:3n3)	6.63 ± 0.83	9.19 ± 2.02 [a]	5.57 ± 0.89 [a]	7.28 ± 0.84 [b]
(20:5n3)	2.15 ± 0.36	1.89 ± 0.40	1.85 ± 0.43 [a]	1.53 ± 0.28 [a]
(22:6n3)	4.81 ± 0.93	7.36 ± 1.73 [a]	6.40 ± 1.01 [a]	12.57 ± 1.29 [a,b]
n-6 PUFAs	75.06 ± 9.31	106.76 ± 15.28 [a]	65.21 ± 10.78 [a]	142.87 ± 13.07 [a,b]
n-3 PUFAs	13.67 ± 1.68	18.43 ± 3.83 [a]	13.57 ± 1.63	21.32 ± 1.81 [a,b]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 2. Polyunsaturated fatty acids (PUFA) content in the FFA fraction (nmol/g of wet tissue) in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	356.30 ± 78.40	495.83 ± 44.51 *	626.91 ± 53.59 *	582.61 ± 59.54 *
(20:4n6)	77.08 ± 9.82	109.19 ± 5.69 *	109.23 ± 5.12 *	153.63 ± 7.87 *,#
(18:3n3)	26.26 ± 5.10	35.10 ± 3.11 *	33.54 ± 4.00 *	31.08 ± 3.65 *
(20:5n3)	3.56 ± 0.82	4.71 ± 0.52 *	3.59 ± 0.62	4.12 ± 0.66 *,#
(22:6n3)	35.16 ± 8.30	46.17 ± 7.06 *	61.57 ± 8.15 *	71.30 ± 8.90 *,#
n-6 PUFAs	433.38 ± 87.32	606.71 ± 48.96 *	736.14 ± 57.79 *	739.06 ± 64.82 *
n-3 PUFAs	64.85 ± 13.92	85.98 ± 8.19 *	99.32 ± 11.71 *	109.76 ± 6.85 *

The data are expressed as mean values ± SD, $n = 10$ in each group. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; # $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Concomitantly, in the DAG fraction, we observed that high-fat diet feeding caused a significant decrease of n-6 PUFAs (LA (−22.2%) and AA (−19.9%)) and n-3 PUFAs (ALA (−22.0%)), whereas the DHA content was markedly elevated (+42.0%) in the white gastrocnemius muscle in comparison with the control group ($p < 0.05$; Table 3). Interestingly, CBD treatment in the rats fed a standard chow resulted in a substantially reduced content of LA (−14.6%, $p < 0.05$, vs. the control group; Table 2) and increased AA (+32.5%, $p < 0.05$, vs. the control group; Table 3) in the white skeletal muscle. However, in the same muscle type, the HFD group after CBD administration was characterized by a significant increment in the pool of LA (+26.8%, $p < 0.05$, vs. the HFD group; Table 3), AA (+130.4%, $p < 0.05$, vs. the control group; +187.5%, $p < 0.05$, vs. the HFD group; Table 3) and DHA (+107.9%, $p < 0.05$, vs. the control group; +46.4%, $p < 0.05$, vs. the HFD group; Table 3), whereas the content of ALA and EPA was considerably reduced (−24.5% and −23.0%, respectively) in comparison with the controls ($p < 0.05$; Table 3). Conversely, in the red gastrocnemius muscle, changes in the total fatty acid content of the DAG fraction in the rats subjected to a high-fat diet were accompanied by a significant increase in the content of n-6 PUFAs (LA (+82.9%) and AA (+18.5%)) as well as all of n-3 PUFAs (ALA (+31.3%), EPA (+23.1%) and DHA (+93.6%)) in comparison with the rats fed a standard diet ($p < 0.05$; Table 4). CBD treatment in the rats fed the standard chow markedly enhanced the AA and EPA content (+29.9% and +39.7%, $p < 0.05$, vs. the control group, respectively; Table 4) with no change in LA, ALA and DHA levels ($p > 0.05$; Table 4) in the oxidative muscle. Similarly, we observed that the HFD-fed group after CBD administration showed a significantly greater accumulation of LA (+70%, $p < 0.05$, vs. the control group; Table 4), AA (+78.8%, $p < 0.05$, vs. the control group; +50.9%, $p < 0.05$, vs. the HFD group; Table 4), ALA (+44.8%, $p < 0.05$, vs. the control group; +10.2%, $p < 0.05$, vs. the HFD group; Table 4), EPA (+80.4%, $p < 0.05$, vs. the control group; +46.5%, $p < 0.05$, vs. the HFD group; Table 4) and DHA (+131.3%, $p < 0.05$, vs. the control group; +19.4%, $p < 0.05$, vs. the HFD group; Table 4) in the pool of the DAG fraction in the red gastrocnemius muscle.

As shown in Table 5, the rats from the HFD group had an increased content of LA (+337.1%), AA (+64.8%), ALA (+138.1%) and DHA (+88.1%) compared to the control group in the white skeletal muscle's TAG fraction ($p < 0.05$; Table 5) with no alterations in the EPA levels ($p > 0.05$; Table 5). Moreover, we noticed that two-week CBD treatment in the rats fed the standard chow considerably elevated only DHA levels (+50.0%, $p < 0.05$, vs. the control group; Table 5) of the TAG fraction in the white skeletal muscle. Concomitantly, in the same muscle type, we observed that CBD administration to rats after the HFD course substantially enhanced the levels of n-6 PUFAs, i.e., of LA and AA (+187.0% and +72.5%, $p < 0.05$, vs. the control group, respectively; Table 5) in the TAG fraction, whereas the LA content in the same experimental group compared to the corresponding untreated HFD

group was decreased (−34.4%, $p < 0.05$; Table 5). Furthermore, we noticed a similar effect of two-week CBD treatment in the case of n-3 PUFAs such as ALA (+95.4%, $p < 0.05$, vs. the control group; Table 5) and DHA (+171.9%, $p < 0.05$, vs. the control group; +43.9%, $p < 0.05$, vs. the HFD group; Table 5), although only the EPA content was significantly reduced (−37.5%, $p < 0.05$, vs. the control group; Table 5) in the white gastrocnemius muscle. With respect to the red skeletal muscle, high-fat feeding considerably intensified the accumulation of LA and DHA (+116.9% and +161.6%, respectively; $p < 0.05$; Table 6) in the TAG fraction in comparison with the rats fed the standard diet. Interestingly, compared to the control conditions, we did not observe any significant alterations in the PUFA composition of the TAG fraction in the CBD group in the same muscle type ($p > 0.05$; Table 6). However, the HFD group after CBD administration exhibited a substantially reduced level of LA (−37.7%, $p < 0.05$, vs. the HFD group; Table 6) along with a substantial rise in n-3 PUFAs EPA (+56.2%, $p < 0.05$, vs. the HFD group; Table 6) and DHA (+249.9%, $p < 0.05$, vs. the control group; +33.7%, $p < 0.05$, vs. the HFD group; Table 6).

Table 3. Polyunsaturated fatty acids (PUFA) content in the DAG fraction (nmol/g of wet tissue) in the white gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	41.10 ± 6.95	35.10 ± 3.93 [a]	31.99 ± 7.50 [a]	40.55 ± 7.49 [b]
(20:4n6)	20.73 ± 2.65	27.46 ± 4.72 [a]	16.61 ± 1.94 [a]	47.76 ± 6.92 [a,b]
(18:3n3)	4.06 ± 0.62	3.73 ± 0.48	3.36 ± 0.86 [a]	3.07 ± 0.31 [a]
(20:5n3)	1.37 ± 0.38	1.06 ± 0.30	1.29 ± 0.31	1.05 ± 0.19 [a]
(22:6n3)	3.68 ± 0.53	3.77 ± 0.71	5.23 ± 0.97 [a]	7.66 ± 0.92 [a,b]
n-6 PUFAs	61.83 ± 9.13	62.56 ± 7.47	48.59 ± 7.61 [a]	90.40 ± 13.61 [a,b]
n-3 PUFAs	9.12 ± 1.23	8.56 ± 1.07	10.05 ± 2.22	12.07 ± 1.58 [a,b]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 4. Polyunsaturated fatty acids (PUFA) content in the DAG fraction (nmol/g of wet tissue) in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD+CBD
(18:2n6c)	79.55 ± 22.95	74.12 ± 5.14	145.53 ± 26.29 *	135.21 ± 18.06 *
(20:4n6)	34.20 ± 5.68	44.42 ± 4.89 *	40.54 ± 2.42 *	61.16 ± 5.46 *,#
(18:3n3)	5.83 ± 1.61	5.82 ± 0.43	7.66 ± 0.51 *	8.45 ± 1.03 *,#
(20:5n3)	0.97 ± 0.18	1.36 ± 0.27 *	1.20 ± 0.61	1.75 ± 0.50 *,#
(22:6n3)	9.09 ± 2.84	9.91 ± 2.02	17.60 ± 2.67 *	21.02 ± 2.85 *,#
n-6 PUFAs	118.71 ± 32.16	118.54 ± 9.37	186.07 ± 26.82 *	202.15 ± 20.80 *
n-3 PUFAs	15.91 ± 4.80	16.81 ± 2.33	26.59 ± 2.56 *	31.22 ± 3.82 *,#

The data are expressed as mean values ± SD, $n = 10$ in each group. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; # $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 5. Polyunsaturated fatty acids (PUFA) content in the TAG fraction (nmol/g of wet tissue) in white gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	306.57 ± 97.30	295.80 ± 47.57	1340.05 ± 383.66 [a]	879.74 ± 181.45 [a,b]
(20:4n6)	17.51 ± 3.63	16.74 ± 2.51	28.85 ± 6.46 [a]	30.21 ± 5.40 [a]
(18:3n3)	31.39 ± 8.98	30.24 ± 5.17	74.72 ± 21.80 [a]	61.33 ± 10.99 [a]
(20:5n3)	4.63 ± 1.58	3.73 ± 1.12	3.95 ± 1.20	2.89 ± 1.48 [a]
(22:6n3)	6.12 ± 2.12	9.18 ± 3.66 [a]	11.56 ± 3.72 [a]	16.64 ± 3.28 [a,b]
n-6 PUFAs	324.08 ± 99.30	313.26 ± 49.97	1290.09 ± 434.82 [a]	909.95 ± 183.75 [a,b]
n-3 PUFAs	42.86 ± 11.48	42.64 ± 8.12	90.98 ± 28.53 [a]	80.19 ± 11.43 [a]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 6. Polyunsaturated fatty acids (PUFA) content in the TAG fraction (nmol/g of wet tissue) in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	775.65 ± 666.51	689.35 ± 226.72	1682.96 ± 820.25 *	1113.21 ± 353.52 [#]
(20:4n6)	38.74 ± 26.02	36.78 ± 9.81	39.97 ± 17.23	39.70 ± 7.85
(18:3n3)	69.66 ± 55.34	66.62 ± 22.17	88.41 ± 37.28	79.79 ± 19.00
(20:5n3)	4.35 ± 1.60	4.15 ± 1.42	3.34 ± 1.02	4.91 ± 1.68 [#]
(22:6n3)	12.72 ± 5.21	13.39 ± 3.49	33.26 ± 6.73 *	44.49 ± 4.76 [*,#]
n-6 PUFAs	965.12 ± 806.91	726.13 ± 236.31	1724.88 ± 837.21 *	1086.75 ± 311.93 [#]
n-3 PUFAs	75.11 ± 49.38	83.67 ± 21.26	136.52 ± 53.96	129.19 ± 19.33

The data are expressed as mean values ± SD, $n = 10$ in each group. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Our study demonstrated that the total fatty acid composition of PL was altered by the high-fat diet in both white and red gastrocnemius muscles. Regarding the glycolytic muscle, we noticed a pronounced decrease in the content of LA, ALA and EPA in the rats subjected to the HFD (−39.5%, −43.9% and −46.8%, respectively; $p < 0.05$; Table 7) compared to the control rats, whereas only the DHA content was increased (+12.7%, $p < 0.05$; Table 7) in the same conditions. CBD given to the rats fed a standard chow did not affect in a statistically significant manner the PUFA content in the white skeletal muscle in the PL fraction. However, when CBD was administered to rats after the HFD course, it considerably reduced the content of LA, ALA and EPA (−27.2%, −37.0% and −37.3%, respectively; $p < 0.05$; Table 7) and simultaneously increased the AA and DHA levels (+21.6% and +69.2%, respectively; $p < 0.05$; Table 7) in comparison with the control conditions. In the same experimental rats, compared to the HFD group alone, we observed a substantially increased accumulation of all PUFAs: LA, AA, ALA, EPA and DHA in the PL fraction (+20.3%, +19.9%, +12.3%, +17.7% and +50.1%, respectively; $p < 0.05$; Table 7). Concomitantly, in the PL fraction of the red gastrocnemius muscle, the HFD-fed rats were characterized by considerably elevated AA and DHA content (+21.5% and +25.6%, respectively; $p < 0.05$; Table 8) with a parallel decline in the levels of ALA and EPA (−42.4% and −30.1%, respectively; $p < 0.05$; Table 8) along with no changes in the LA content

($p > 0.05$; Table 8) compared to the controls. CBD administration while feeding rats with the standard chow resulted in a pronounced increase in n-6 PUFAs, that is, LA (+21.0%) and AA (+19.4%) as well as in n-3 PUFAs, that is, ALA (+11.1%) and DHA (+53.3%) ($p < 0.05$; Table 8) in comparison with the control group. Additionally, we observed a significant increase in the PUFA content of the PL fraction after prolonged CBD administration in the high-fat diet rats: LA (+27.2%, $p < 0.05$, vs. the control group; +23.1%, $p < 0.05$, vs. the HFD group), AA (+53.4%, $p < 0.05$, vs. the control group; +26.3%, $p < 0.05$, vs. the HFD group) and DHA (+78.7%, $p < 0.05$, vs. the control group; +42.2%, $p < 0.05$, vs. the HFD group) in the red skeletal muscle (Table 8). These CBD effects were accompanied by a reduction in the ALA and EPA content (−32.7% and −25.9%, respectively; $p < 0.05$; Table 8) compared to the control rats in the red skeletal muscle.

Table 7. Polyunsaturated fatty acids (PUFA) content in the PL fraction (nmol/g of wet tissue) in the white gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	3804.57 ± 232.32	3562.35 ± 603.79	2302.71 ± 204.26[a]	2770.34 ± 572.05 [a,b]
(20:4n6)	4249.78 ± 200.32	4074.72 ± 448.51	4168.76 ± 294.02	5146.26 ± 568.67 [a,b]
(18:3n3)	45.76 ± 6.35	47.49 ± 3.80	25.67 ± 2.46 [a]	28.83 ± 2.45 [a,b]
(20:5n3)	49.26 ± 5.52	46.56 ± 5.41	26.23 ± 3.09 [a]	30.88 ± 5.25 [a,b]
(22:6n3)	1563.04 ± 119.25	1698.80 ± 44.15	1761.42 ± 265.15 [a]	2644.32 ± 420.35 [a,b]
n-6 PUFAs	8054.35 ± 342.47	7925.37 ± 456.35	6471.47 ± 344.45 [a]	7622.72 ± 647.02 [b]
n-3 PUFAs	1658.06 ± 122.19	1877.10 ± 694.05	1813.33 ± 266.27	2801.95 ± 712.31 [a,b]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 8. Polyunsaturated fatty acids (PUFA) content in the PL fraction (nmol/g of wet tissue) in the red gastrocnemius muscle in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
(18:2n6c)	6016.99 ± 705.69	7281.82 ±495.03 *	6140.77 ± 331.08	7653.15 ± 440.06 [*,#]
(20:4n6)	3922.92 ± 371.42	4684.31 ±184.04 *	4765.12 ± 384.66 *	6019.30 ± 184.80 [*,#]
(18:3n3)	71.94 ± 7.37	79.89 ± 2.95 *	41.45 ± 4.01 *	48.43 ± 6.68 [*,#]
(20:5n3)	58.99 ± 5.93	54.48 ± 4.28	41.21 ± 3.97 *	43.74 ± 2.36 *
(22:6n3)	2283.89 ± 382.17	3793.13 ± 419.76 *	3052.58 ± 291.44 *	4341.88 ± 260.71 [*,#]
n-6 PUFAs	9939.90 ± 907.86	11,884.97 ± 503.74 *	11,008.32 ± 512.03 *	13,672.45 ± 592.99 [*,#]
n-3 PUFAs	2419.60 ± 397.65	3857.01 ± 450.31 *	3140.37 ± 286.37 *	4471.28 ± 250.34 [*,#]

The data are expressed as mean values ± SD, $n = 10$ in each group. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

In the plasma FFA fraction of the rats fed an HFD, we noticed significantly increased levels of n-6 PUFAs, i.e., of LA (+68.5%) and AA (+107.3%) and of n-3 PUFA DHA (+31.8%) ($p < 0.05$; Table 9). Interestingly, CBD administration to the animals fed a standard diet caused a pronounced elevation of all the PUFAs: LA, AA, ALA, EPA and DHA (+52.3%, +88.2%, +43.6%, +35.0% and +108.1%, respectively; $p < 0.05$; Table 9) in comparison with the corresponding control rats. Similarly, the high fat diet-fed rats after CBD administration presented a significantly elevated level of LA (+167.9%, $p < 0.05$, vs. the control group; +59.0%, $p < 0.05$, vs. the HFD group), AA (+90.5%, $p < 0.05$, vs. the control group), ALA

(+95.5%, $p < 0.05$, vs. the control group; +67.2%, $p < 0.05$, vs. the HFD group) and DHA (81.2%, $p < 0.05$, vs. the control group; +37.5%, $p < 0.05$, vs. the HFD group). The only fatty acid of plasma FFA PUFAs with the content reduced in the rats fed an HFD after two-week CBD treatment was EPA (−23.6%, $p < 0.05$, vs. the control group; Table 9). However, in comparison with the corresponding untreated HFD group, the EPA content was substantially enhanced (+33.5%, $p < 0.05$; Table 9). On the other hand, in the plasma TAG fraction, the high-fat diet resulted in a considerable decrease in fatty acids belonging to n-3 PUFAs: ALA (−46.6%), EPA (−65.8%) and DHA (−56.1%) ($p < 0.05$, vs. the control group; Table 10). Moreover, we observed a marked elevation of the DHA content in the plasma TAG fraction of animals fed a standard diet after prolonged CBD administration (+22.9%, $p < 0.05$; Table 10) compared to the controls. Consistently, CBD given to the rats after the HFD course significantly reduced the EPA and DHA content (−61.5% and −47.4%, $p < 0.05$, vs. the control group; Table 10). However, in comparison with the HFD group alone, we reported in the same experimental group a substantially declined content of AA in the chronic presence of CBD (−18.4%, $p < 0.05$; Table 10) and, conversely, a significantly greater content of ALA (+60.0%, $p < 0.05$; Table 10).

Table 9. Polyunsaturated fatty acids (PUFA) content in the plasma FFA fraction (nmol/mL of plasma) in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
18:2n6c	55.06 ± 12.31	83.88 ± 23.49 [a]	92.76 ± 25.91 [a]	147.53 ± 40.28 [a,b]
20:4n6	8.16 ± 1.68	15.36 ± 2.17 [a]	16.37 ± 4.24 [a]	17.34 ± 4.88 [a]
18:3n3	6.39 ± 1.75	9.18 ± 2.54 [a]	7.48 ± 1.73	12.50 ± 3.72 [a,b]
20:5n3	0.40 ± 0.08	0.49 ± 0.13 [a]	0.23 ± 0.05 [a]	0.29 ± 0.08 [a,b]
22:6n3	2.14 ± 0.58	4.30 ± 1.16 [a]	2.82 ± 0.67 [a]	3.53 ± 1.28 [a,b]
n-6 PUFAs	63.22 ± 13.49	100.93 ± 26.71 [a]	109.13 ± 28.51 [a]	157.30 ± 40.34 [a,b]
n-3 PUFAs	8.93 ± 2.35	14.49 ± 3.78 [a]	10.14 ± 2.58	16.32 ± 4.81 [a,b]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Table 10. Polyunsaturated fatty acids (PUFA) content in the plasma TAG fraction (nmol/mL of plasma) in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment.

Fatty Acid	Control	CBD	HFD	HFD + CBD
18:2n6c	1746.27 ± 258.44	1633.25 ± 445.53	1660.73 ± 964.61	1764.09 ± 408.46
20:4n6	126.75 ± 19.27	136.13 ± 19.36	132.11 ± 24.99	107.80 ± 18.37 [b]
18:3n3	183.83 ± 40.07	176.14 ± 66.09	133.72 ± 95.65 [a]	151.86 ± 53.02 [b]
20:5n3	32.35 ± 6.81	32.34 ± 7.05	11.10 ± 2.69 [a]	12.49 ± 3.11 [a]
22:6n3	105.50 ± 21.13	129.68 ± 18.44 [a]	46.35 ± 8.78 [a]	55.49 ± 16.04 [a]
n-6 PUFAs	1880.31 ± 264.08	1689.88 ± 377.78	2130.27 ± 992.20	1882.45 ± 426.13
n-3 PUFAs	327.95 ± 56.27	340.65 ± 71.39	151.91 ± 40.00 [a]	221.56 ± 70.30 [a,b]

The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD; 18:2n6c (linoleic acid, LA); 20:4n6 (arachidonic acid, AA); 18:3n3 (α-linolenic acid, ALA); 20:5n3 (eicosapentaenoic acid, EPA); 22:6n3 (docosahexaenoic acid, DHA).

Our study demonstrated that the high-fat diet substantially reduced the n-6/n-3 PUFA ratio in the FFA and DAG fractions (−14.2% and −27.6%, $p < 0.05$; Figure 1A,B, respectively) in the white gastrocnemius muscle compared to the controls, whereas CBD administration considerably enhanced it (+41.7% and +49%.0%, $p < 0.05$; Figure 1A,B, respectively) in comparison with the HFD group alone. Regarding the FFA fraction, we also found that two-week CBD treatment significantly increased the n-6/n-3 PUFA ratio in the rats fed either the standard chow or the high-fat diet in the white gastrocnemius muscle (+11.6% and +21.6%, $p < 0.05$; Figure 1A) in comparison with the control group. On the other hand, in the red gastrocnemius muscle, we observed elevated n-6/n-3 PUFA ratio in the rats fed HFD in the FFA fraction (+12.9%, $p < 0.05$, vs. the control group; Figure 1A), whereas chronic CBD treatment resulted in a significant decrease in the same experimental group (−9.3%, $p < 0.05$, vs. the HFD group; Figure 1A). Interestingly, in the red gastrocnemius muscle, we observed a substantial reduction in the n-6/n-3 PUFA ratio in the rats subjected to standard chow or high-fat diet feeding after CBD administration in the DAG (−6.6% and −13.7%, $p < 0.05$, vs. the control group, respectively; −11.6%, $p < 0.05$, vs. the HFD group; Figure 1B) and PL fractions (−21.0% and −21.2%, $p < 0.05$, vs. the control group, respectively; −13.1%, $p < 0.05$, vs. the HFD group; Figure 1D). With respect to the TAG fraction (Figure 1C) in the white and red gastrocnemius muscles, we observed that the HFD-fed groups exhibited a significantly increased n-6/n-3 PUFA ratio (+66.8% and +28.1%, respectively; $p < 0.05$; Figure 1C) in comparison with the control group. Concomitantly, two-week CBD treatment caused a substantial reduction in the n-6/n-3 PUFA ratio (−14.9% and −30.1%, respectively; $p < 0.05$; Figure 1C) compared to the HFD group alone. Moreover, the n-6/n-3 PUFA ratio in the TAG fraction only in the white gastrocnemius muscle was considerably reduced by CBD administration in the rats fed a standard chow (−9.5%, $p < 0.05$; Figure 1C) and, conversely, in the rats fed the HFD, it was significantly elevated (+42.0%, $p < 0.05$; Figure 1C) in comparison with the control group. Simultaneously, in the PL fraction, we observed that the HFD-fed groups exhibited a markedly decreased n-6/n-3 PUFA ratio in both the white (−23.8%, $p < 0.05$; Figure 1D) and the red (−9.3%, $p < 0.05$; Figure 1D) gastrocnemius muscle compared to the control conditions. Moreover, CBD administration to animals being on an HFD in the white gastrocnemius muscle resulted in a pronounced reduction of the n-6/n-3 PUFA ratio in the PL fraction (−13.9%, $p < 0.05$, vs. the HFD group; Figure 1D).

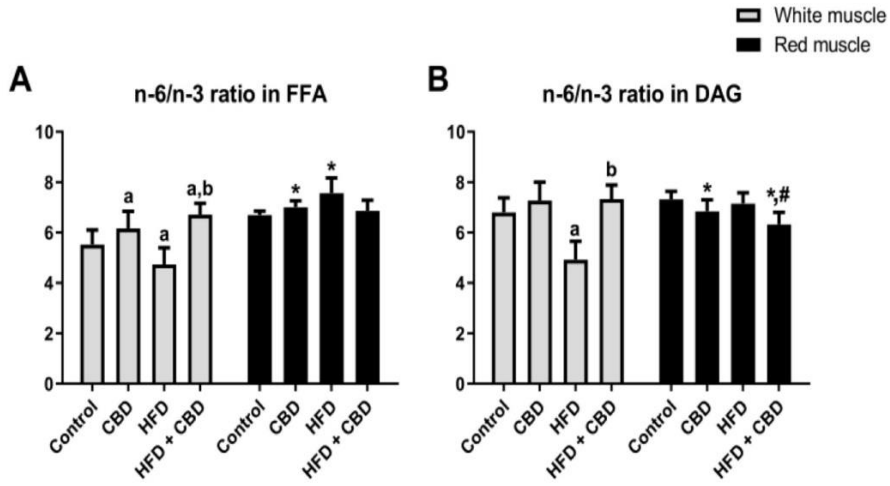


Figure 1. *Cont.*

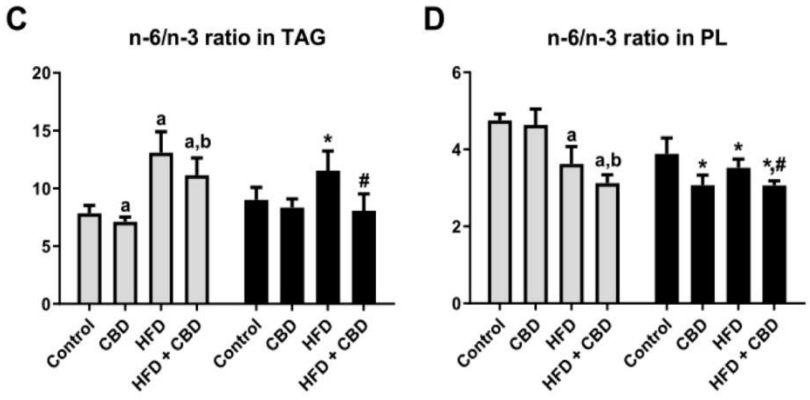


Figure 1. Intramuscular n-6/n-3 PUFA ratio in the (**A**) free fatty acid (FFA), (**B**) diacylglycerol (DAG), (**C**) triacylglycerol (TAG) and (**D**) phospholipid (PL) fractions in the white and red gastrocnemius muscles in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment. The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle.

Induction of obesity by high-fat diet feeding resulted in a significantly increased n-6/n-3 PUFA ratio in both HFD-fed groups (untreated and treated with CBD) in the plasma FFA (+47.3% and +37.3%, respectively; $p < 0.05$; Figure 2A) and TAG fractions (+57.1% and +49.1%, respectively, $p < 0.05$; Figure 2B) compared to the control rats. Furthermore, in the plasma of the rats fed a standard diet and injected with CBD, we observed a markedly decreased n-6/n-3 PUFA ratio in the FFA (−7.4%, $p < 0.05$; Figure 2A) and TAG (−6.9%, $p < 0.05$; Figure 2B) fractions in comparison with the control group. Importantly, the n-6/n-3 PUFA ratio was substantially decreased in the chronic presence of CBD during the high-fat diet feeding course in the plasma FFA fraction (−6.8%, $p < 0.05$, vs. the HFD group; Figure 2A).

3.2. Effect of Two-Week CBD Treatment on the Oxidative and Antioxidative Parameters in the White and Red Skeletal Muscles of Rats Subjected to Standard and High-Fat Diets

In the experimental model of HFD-induced obesity, we noticed significantly decreased CAT values after two-week CBD administration in the rats fed the high-fat diet (−12.8%, $p < 0.05$, vs. the HFD group; Figure 3A) only in the red gastrocnemius muscle. Concomitantly, we did not observe any changes in catalase concentrations in the white gastrocnemius muscle ($p > 0.05$; Figure 3A). Additionally, two-week CBD treatment of the rats fed a standard or high-fat diet resulted in a pronounced increase in SOD2 concentrations (+9.9% and +8.8%, respectively; $p < 0.05$; Figure 3B) in the white gastrocnemius muscle in comparison with the controls. Similar effects of CBD administration we reported in the red gastrocnemius muscle in the rats fed a standard chow (+7.4%, $p < 0.05$, vs. the control group; Figure 3B). Importantly, in both white and red skeletal muscles, chronic CBD administration caused a considerable increment in SOD2 levels in the rats subjected to the high-fat diet (+7.1% and +3.9%, $p < 0.05$; Figure 3B) compared to the HFD group alone. With respect to the total antioxidant capacity, we observed a substantial increase in the HFD-fed group in the chronic presence of CBD (+9.1%, $p < 0.05$, vs. the control group; Figure 3C) only in the white skeletal muscle. Moreover, in the same muscle, in the standard chow-fed group, after CBD treatment, we observed a trend towards an increase in the TAC ($p = 0.0661$; Figure 3C). Simultaneously, we did not notice any significant alterations in

TAC levels in the red gastrocnemius muscle ($p > 0.05$; Figure 3C). Furthermore, as expected, the high-fat diet feeding induced a greater intramuscular AGE concentration in both white and red skeletal muscles (+13.3% and +32.1%, respectively; $p < 0.05$, vs. the control group; Figure 3D), which was subsequently decreased by chronic CBD administration (−38.8% and −28.0%, respectively; $p < 0.05$, vs. the HFD group; Figure 3D). A similar effect of CBD treatment was also observed in the rats fed a standard diet (−64.5%, $p < 0.05$; Figure 3D) in the white gastrocnemius muscle compared to the control group. Consequently, the MDA content was significantly increased in the animals fed an HFD in the red gastrocnemius muscle (+45.6%, $p < 0.05$, vs. the control group; Figure 3E), which was further reduced after CBD administration (−26.9% $p < 0.05$, vs. the HFD group; Figure 3E). Regarding the white skeletal muscle, the MDA concentration was considerably decreased in the presence of CBD during high-fat diet administration (−16.7%, $p < 0.05$, vs. the control group; −22.9%, $p < 0.05$, vs. the HFD group; Figure 3E). Similarly, high-fat diet feeding resulted in a substantial increase in the 4-HNE levels in both white and red gastrocnemius muscles (+43.7% and +115.7%, respectively; $p < 0.05$; Figure 3F) in comparison with the control conditions. In addition, we noticed a significantly decreased content of 4-HNE after prolonged CBD treatment in the rats fed a standard chow (−39.8%, $p < 0.05$, vs. the control group; Figure 3F) in the white gastrocnemius muscle, whereas, on the contrary, we observed a pronounced increase in the 4-HNE concentration in the same experimental group in the red skeletal muscle (+124.6%, $p < 0.05$; Figure 3F) compared to the control rats.

3.3. Effect of Two-Week CBD Treatment on the Total Intramuscular Expression of Proteins Involved in the Inflammatory Pathway in the White and Red Skeletal Muscles of Rats Subjected to Standard and High-Fat Diets

Compared to the control group, the rats fed a high-fat diet were characterized by a substantial increase in the total expression of COX2 and 5-LO in the red gastrocnemius muscle (+22.3% and +8.9%, respectively; $p < 0.05$; Figure 4B,C). Moreover, during HFD administration, we observed a trend towards an increase in the COX1 expression ($p = 0.0590$, vs. the control group; Figure 4A) in the white gastrocnemius muscle. Most importantly, two-week CBD injections in the high-fat diet group resulted in a considerable reduction of the total intramuscular expression of the proteins involved in the inflammatory pathway in both white and red gastrocnemius muscles, i.e., of COX1 (−49.5% and −39.0%, respectively; $p < 0.05$; Figure 4A) and COX2 (−39.5% and −28.4%, respectively; $p < 0.05$; Figure 4B) compared to the HFD alone. Similar effects of CBD treatment in the HFD-fed rats we reported in the case of the total intramuscular 5-LO expression in the white gastrocnemius muscle (−19.1%, $p < 0.05$, vs. the control group; −26.9%, $p < 0.05$, vs. the HFD group; Figure 4C). Simultaneously, we noticed a markedly declined total expression of COX1 and 5-LO in the rats fed a standard chow with the chronic presence of CBD in the red skeletal muscle (−32.8% and −17.5%, $p < 0.05$, vs. the control group; Figure 4A,C). Concomitantly, in the white skeletal muscle, we observed that CBD treatment of the rats subjected to an HFD substantially increased the total expression of anti-inflammatory 15-LO (+43.2%, $p < 0.05$; Figure 4D) in comparison with the HFD group alone. As presented in Figure 4, intramuscular PPARγ expression decreased considerably during the course of a high-fat diet in the white (−28.5%, $p < 0.05$; Figure 4E) and red (−28.8%, $p < 0.05$; Figure 4E) skeletal muscles in comparison with the control rats. However, prolonged CBD administration in the high-fat diet rats resulted in pronounced restoration of the total expression of PPARγ in both muscle types (+44.2% and +25.0%, respectively; $p < 0.05$; Figure 4E) compared to the HFD group. Furthermore, the group receiving a high-fat diet demonstrated a significant elevation in the total muscular expression of NF-κB in the red gastrocnemius muscle (+81.5%, $p < 0.05$, vs. the control group; Figure 4F). We also found that in the same muscle type, chronic CBD treatment in the HFD-fed rats substantially reduced the expression of NF-κB (−39.4%, $p < 0.05$; Figure 4F) and IL-6 (−17.2%, $p < 0.05$; Figure 5B) in comparison with the HFD group. Similarly, in the white gastrocnemius muscle of the rats subjected to the standard or high-fat chow administration, we observed a significant decrease in the total expression of NF-κB after CBD injections (−27.8% and −35.8%, respectively,

$p < 0.05$, vs. the control group; −30.3%, $p < 0.05$, vs. the HFD group; Figure 4F) and IL-6 (−34.7% and −39.7%, respectively, $p < 0.05$, vs. the control group; −29.9%, $p < 0.05$, vs. the HFD group; Figure 5B). In the case of TNF-α, we reported that the rats subjected to an HFD presented a substantially increased expression (+26.6%, $p < 0.05$, vs. the control group; Figure 5A), which was further decreased by CBD treatment (−18.7%, $p < 0.05$, vs. the HFD group; Figure 5A) in the white gastrocnemius muscle. Concomitantly, we did not notice any significant changes in TNF-α expression in the oxidative muscle ($p > 0.05$; Figure 5A). Moreover, we observed that high-fat diet feeding reduced the total expression of Nrf2 in the red skeletal muscle (−25.3%, $p < 0.05$; Figure 5C) and Bcl-2 in both muscle types (−21.5% and −31.3%, respectively; $p < 0.05$; Figure 5D) compared to the controls. Interestingly, Nrf2 expression considerably increased in the standard chow-fed group after two-week CBD injections (+30.1%, $p < 0.05$, vs. the control group; Figure 5C) in the white gastrocnemius muscle. Similar effects of CBD treatment compared to the control conditions were observed with respect to the total expression of Bcl-2 (+38.1%, $p < 0.05$; Figure 5D) in the red skeletal muscle. Concomitantly, we also noticed a substantial increase in the total Bcl-2 expression in the white and red gastrocnemius muscles of the HFD group after CBD administration (+29.5% and +50.9%, respectively; $p < 0.05$; Figure 5D) in comparison with the HFD group alone. Moreover, in both muscle types, we reported that high-fat diet feeding significantly increased the total expression of MMP-2 (+37.9%, $p < 0.05$; Figure 5E) and MMP-9 (+19.1% and +27.2%, respectively; $p < 0.05$; Figure 5F) compared to the respective controls. Simultaneously, in the red gastrocnemius muscle of the rats subjected to the standard or high-fat chow administration, we observed a significant decrease in the total expression of MMP-2 after CBD administration (−21.6% and −21.8%, respectively, $p < 0.05$, vs. the control group; −33.2%, $p < 0.05$, vs. the HFD group; Figure 5E). Most importantly, CBD injections in the HFD-fed rats considerably decreased the expression of MMP-9 in both white (−21.5%, $p < 0.05$; Figure 5F) and red (−16.4%, $p < 0.05$; Figure 5F) gastrocnemius muscles in comparison with the HFD group.

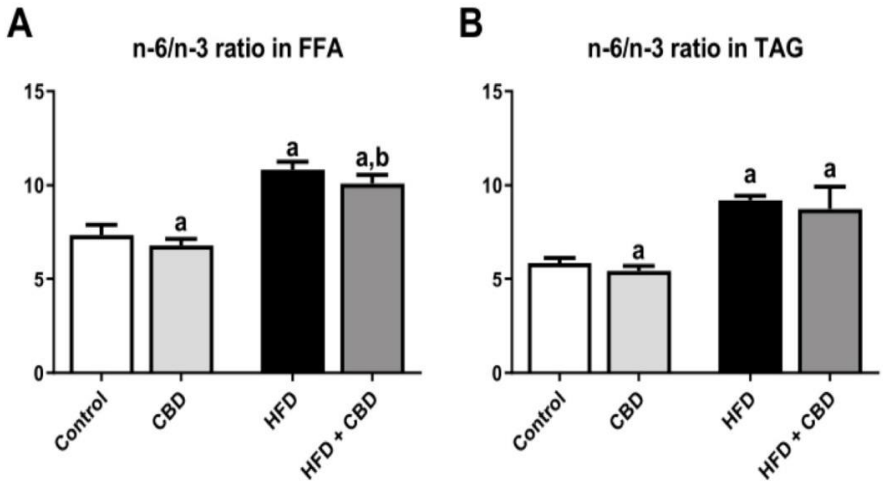


Figure 2. Plasma n-6/n-3 PUFA ratio in the (**A**) free fatty acid (FFA) and (**B**) triacylglycerol (TAG) fractions in the control (standard diet) and high-fat diet (HFD) groups after two-week cannabidiol (CBD) treatment. The data are expressed as mean values ± SD, $n = 10$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD.

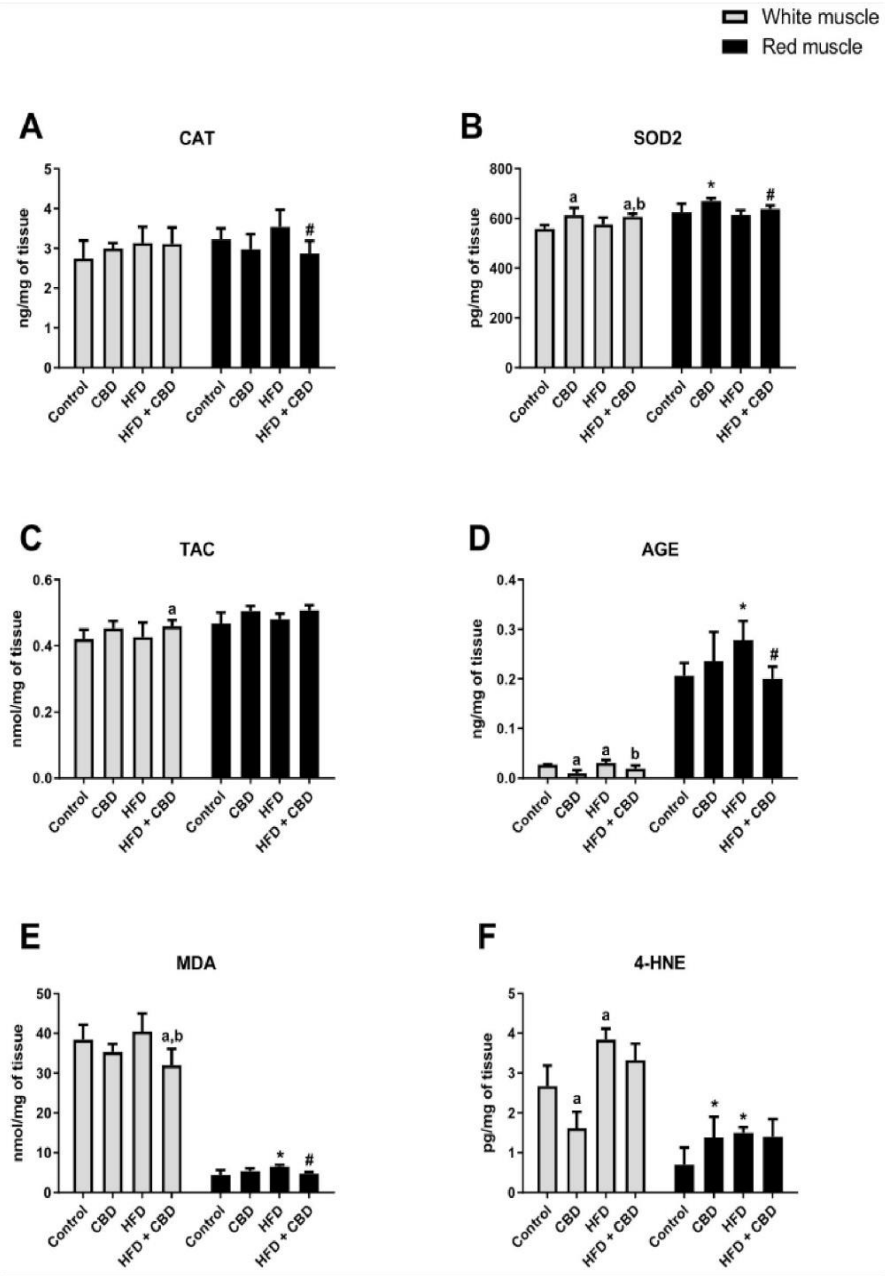


Figure 3. The effect of two-week cannabidiol (CBD) treatment on oxidative stress parameters, i.e., on (**A**) catalase (CAT), (**B**) superoxide dismutase 2 (SOD2), (**C**) total antioxidant capacity (TAC), (**D**) advanced glycation end products (AGE), (**E**) malonyldialdehyde (MDA) and (**F**) 4-hydroxynonenal (4-HNE), in the white and red gastrocnemius muscles in the control (standard diet) and high-fat diet (HFD) groups. The data are expressed as mean values ± SD, $n = 6$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle.

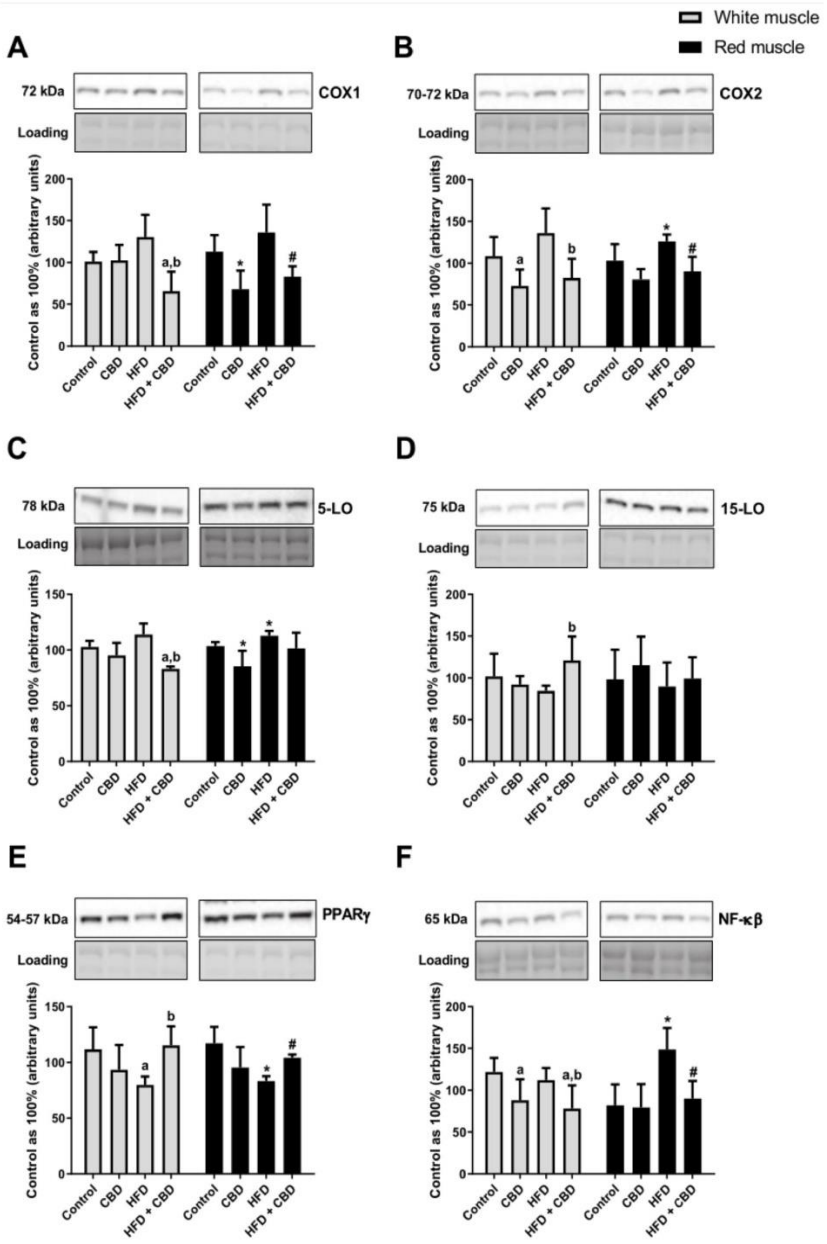


Figure 4. The total expression of proteins involved in the eicosanoid synthesis pathway, i.e., of (**A**) cyclooxygenase-1 (COX-1), (**B**) cyclooxygenase-2 (COX-2), (**C**) 5-lipoxygenase (5-LO), (**D**) 15-lipoxygenase (15-LO) and (**E**) peroxisome proliferator-activated receptor gamma (PPARγ), as well as total expression of (**F**) nuclear factor-κB (NF-κB) in the white and red gastrocnemius muscles in the control (standard diet) and high-fat diet (HFD) groups. The total expressions of the abovementioned proteins are presented as percentage differences compared to the control group which was set as 100%. The data are expressed as mean values ± SD, $n = 6$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle. * $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle.

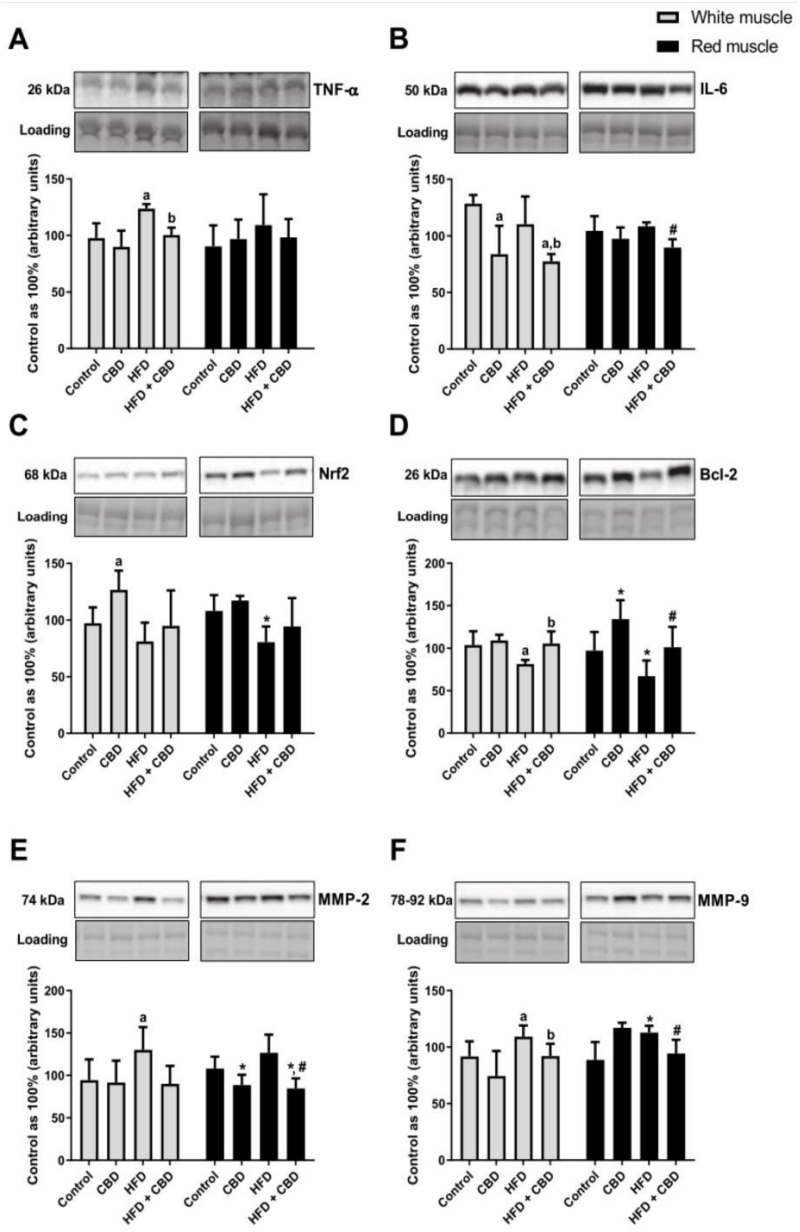


Figure 5. Intramuscular changes in the total expression of proteins involved in the inflammatory pathway, i.e., of (**A**) tumor necrosis factor α (TNF-α), (**B**) interleukin 6 (IL-6), as well as (**C**) nuclear factor erythroid 2-related factor 2 (Nrf2), (**D**) B cell lymphoma 2 (Bcl-2), (**E**) matrix metalloproteinase-2 (MMP-2) and (**F**) matrix metalloproteinase-9 (MMP-9) in the white and red gastrocnemius muscles in the control (standard diet) and high-fat diet (HFD) groups. The total expressions of the abovementioned proteins are presented as percentage differences compared to the control group which was set as 100%. The data are expressed as mean values ± SD, $n = 6$ in each group. [a] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the white gastrocnemius muscle; [b] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the white gastrocnemius muscle. [*] $p < 0.05$ indicates a significant difference: the control group vs. the examined group in the red gastrocnemius muscle; [#] $p < 0.05$ indicates a significant difference: HFD vs. HFD + CBD in the red gastrocnemius muscle.

4. Discussion

Our research has shown that an obesogenic high-fat diet influences the fatty acid composition in the white and red skeletal muscles as well as contributes to the development of oxidative stress and local inflammation. Moreover, for the first time, we evaluated the impact of CBD on the n-6/n-3 PUFA ratio in different lipid fractions together with oxidative stress parameters and inflammatory pathways in a rat model of HFD-induced obesity. It is worth emphasizing, that the present study assessed the effect of CBD on the abovementioned levels in relation to the metabolism and oxidative capacity of the two types of skeletal muscle tissues (namely, with dominant oxidative vs. glycolytic metabolism).

Our data showed that HFD feeding caused an imbalance in the n-6/n-3 PUFA ratio, which was further effectively improved by two-week CBD administration. Specifically, a shift in the n-6/n-3 PUFA equilibrium in favor of anti-inflammatory n-3 PUFAs, especially in the red gastrocnemius muscle, which is probably due to the fact that the fibers of this type use FAs as an energy substrate for oxidative metabolism. In recent years, several studies have reported that n-6 and n-3 PUFAs have an opposite effect on IR and body homeostasis [33,34]. It is postulated that n-3 PUFAs, including ALA (C18:3; a precursor of EPA), EPA (C20:5) and DHA (C22:6), attenuate the development of IR via reducing inflammation, while n-6 PUFAs, primarily AA (C20:4) and its precursor LA (C18:2), promote emergence of IR [35]. The latest evidence also indicates that especially AA plays a key role in the inflammatory process and is associated with various metabolic diseases [11]. Therefore, it seems reasonable to control the dietary ratios of n-6/n-3 PUFAs in order to ameliorate obesity-related IR, which is beneficial for protection against chronic and metabolic diseases. In our study, as expected, we demonstrated that feeding rats an HFD resulted in an increased accumulation of fatty acids in the red gastrocnemius muscle in various lipid fractions (i.e., FFA, DAG, TAG and PL) with a concomitant shift in the n-6/n-3 PUFA balance towards n-6 PUFAs, mostly due to the elevated content of LA and AA. On the other hand, in the case of the white gastrocnemius muscle, we observed similar effects only in the TAG fraction, whereas in the FFA, DAG and PL lipid pools, there was a slight reduction in the n-6/n-3 PUFA ratio. Increased fatty acids accumulation in the TAG fraction results from the fact that this lipid pool serves as an energy substrate store [36]. In the red gastrocnemius muscle, the increase of the n-6/n-3 levels in response to HFD feeding turned out to be more pronounced than in the white type, which is consistent with the metabolism and use of FAs as an energy substrates by oxidative fibers [37]. Simultaneously, lipid oversupply induced an elevation in the proportion of circulating n-6 PUFAs to n-3 PUFAs in both plasma FFA and TAG, which was in line with the findings of other researchers [38]. Interestingly, in our experiment, we found that the observed increase in the ratio of n-6 to n-3 PUFAs in the plasma FFA fraction resulted from a significant increase in the LA and AA contents, whereas with regard to plasma TAG, this shift was due to marked reduction in the amount of n-3 PUFAs, i.e., of ALA, EPA and DHA. However, the major finding of the present study is that two-week CBD treatment could effectively lower the n-6/n-3 PUFA ratio by shifting the equilibrium in favor of n-3 fatty acids and increasing their incorporation into different lipid fractions. In the red gastrocnemius muscle, we noticed significantly elevated n-3 PUFAs, mainly EPA and DHA, along with a substantial reduction in the LA content (n-6 PUFA) in all the examined lipid fractions of the animals chronically treated with CBD during an HFD course. It is worth noting that tissue LA is considered an obesity-promoting FA since it serves as a precursor to AA, which is a substrate in the synthesis of AEA and 2-AG [39]. Thus, the increased synthesis of these ECs largely explains the ability of LA to produce obesogenic effects. Moreover, in our study, we revealed that the AA concentration was significantly enhanced in the FFA, DAG and PL lipid pools in the rats fed both the standard chow and the HFD after CBD injections in the muscle with oxidative metabolism, which may have resulted from the overactivation of the eCBome in obesity and its additional stimulation by CBD [40]. Surprisingly, in the white skeletal muscle, CBD caused a pronounced elevation in the n-6/n-3 PUFA ratio in the FFA and DAG fractions of the rats fed an HFD resulting from increased incorporation of LA and

AA. Conversely, in the TAG and PL lipid pools of the same muscle type, we observed an improvement in the n-6/n-3 PUFA imbalance after CBD administration, which was associated with a greater increase in the content of essential n-3 PUFA species, mainly, the amount of DHA. These findings demonstrate that CBD may play a pivotal role in the control of the membrane fatty acid content through the reduction of substrates for ECs and inflammatory molecules synthesis as well as improvement in the tissue n-3 essential fatty acids concentration. Additionally, an increasing amount of evidence suggests that n-3 PUFAs improve insulin sensitivity in skeletal muscle and liver in different animal models, e.g., in diet-induced obese mice and rats [41–44].

Moreover, the following study showed a link between the shifts in the n-6/n-3 PUFA balance towards n-6 PUFAs and the increasing inflammatory response in fatty acid oversupply conditions. N-6 PUFAs, particularly AA, are considered to be the most important source of precursors for the synthesis of ECs and proinflammatory eicosanoids, including prostaglandins, leukotrienes, thromboxanes and hydroxyeicosatetraenoic acids (HETEs), which intensify the inflammatory signaling cascade [45]. Consistently, in our research, we observed a substantial elevation in the total expression of enzymes catalyzing the production of the abovementioned lipid mediators, such as cyclooxygenase 1 (COX1), cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LO) in both white and red gastrocnemius muscles of the rats subjected to an HFD for seven weeks. Simultaneously, two-week CBD administration significantly reduced their skeletal muscle expression in the same HFD group, which is in line with previous findings describing the inhibitory action of CBD on COX1, COX2 and 5-LO in an in vitro model (human colon adenocarcinoma cell line HT29) [46,47]. Additionally, in the white skeletal muscle, CBD treatment caused a substantial elevation of anti-inflammatory 15-lipoxygenase (15-LO) in the HFD-fed rats [48]. Furthermore, in our study, we noticed a considerably elevated PPARγ expression after CBD administration in the rats fed an HFD regardless of the metabolism of a given muscle (oxidative vs. glycolytic). We can assume that the decreased expression of proinflammatory enzymes in muscles after CBD injections may be indirectly related to an increase in the expression of PPARγ, whose CBD is an agonist [49]. Moreover, some studies have shown that n-3 PUFAs can increase PPARγ expression in vitro (e.g., the HaCaT keratinocyte cell line [50]) and in vivo (e.g., C57BL/6 mice [51]) as natural ligands of this transcription factor; therefore, we can hypothesize that anti-inflammatory properties of n-3 PUFAs may be related to the PPARγ upregulation. Accordingly, Hou et al. demonstrated that PPARγ activation results in proteasomal degradation of the p65 subunit of nuclear factor-κB (NF-κB) which inhibits expression of the genes involved in inflammation (e.g., COX2) as well as of some proinflammatory mediators (e.g., TNF-α and IL-6) [52]. The results obtained in our experiment are in agreement with this statement; we noticed a pronounced reduction in the total muscular expression of NF-κB after two-week CBD administration in both white and red gastrocnemius muscles of high-fat diet-fed rats in comparison with the HFD group alone. Concomitantly, similar alterations caused by CBD in the expression of inflammatory mediators, such as IL-6, were noticeable in the muscles with predominant oxidative and glycolytic metabolism of the rats subjected to a high-fat diet. Moreover, PPARγ also collaborates with another transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates many cytoprotective genes such as antioxidant proteins, including CAT and SOD2 (Scheme 1) [53,54]. In the studies conducted by Huang et al., the researchers revealed that PPARγ was activated by transcriptional activity of Nrf2, which was also confirmed by several other studies [55,56]. However, some lines of evidence indicated the possibility of direct activation of the Nrf2 pathway by PPARγ, which suggests that the Nrf2 and PPARγ pathways are related in a positive feedback loop [57]. Our research revealed that high-fat diet feeding in rats decreased the expression of Nrf2, which was further restored by two-week CBD administration. Intriguingly, CBD also elevated the total Nrf2 expression in the rats fed a standard chow; however, changes reached the significant level only in the white gastrocnemius muscle. In in vitro studies, Mammana et al. also displayed that CBD in a dose of 2.5 and 5 μM substantially upregulated the expression of Nrf2 in

NSC-34 motor neurons with lipopolysaccharide (LPS)-induced inflammation [58]. In the same experiment, the researchers pointed out a considerable elevation in expression of B cell lymphoma 2 (Bcl-2), an anti-apoptotic protein, following CBD treatment (5 μM), which is consistent with the results obtained in our experiment, where we observed a significant increase in the Bcl-2 protein in both muscle types in the rats fed an HFD [58]. Additionally, in our study, we reported that the rats subjected to high-fat diet feeding exhibited a substantially increased muscular expression of matrix metalloproteinases (MMPs) MMP-2 and MMP-9 which belong to the enzymes that promote the breakdown and remodeling of tissues as well as the inflammatory process and related migration of white blood cells [59]. It is worth emphasizing that two-week CBD administration considerably decreased the total MMP-2 and MMP-9 expression in the same experimental animals in both white and red gastrocnemius muscles. Similar results were obtained by Elbaz et al. in a study where CBD treatment (6 μM) reduced the activities of MMP-2 and MMP-9 in triple-negative breast cancer (TNBC) cell lines [60]. This finding further implies CBD's potentially beneficial cellular protective effects.

In obesity, the excessive amount of fat exceeds the ability of skeletal muscles to oxidize this energy substrate, which leads to the development of lipotoxicity and disruption of the physiological muscle function, including impaired insulin signaling. These harmful effects of accumulated lipid intermediates were partially attributed to the ROS/RNS overproduction that, together with attenuated antioxidant defense capacity, elicits systemic oxidative stress [61]. It is worth noting that skeletal muscles, due to different fiber type composition (oxidative vs. glycolytic), vary in susceptibility to oxidative stress-induced damage since they have different contents of mitochondria that generate ROS and distinct antioxidative capacity [62]. Myocytes have their own antioxidant system containing various antioxidant enzymes including CAT, SOD2 and GPx [62]. In the present study, we reported that feeding rats an HFD did not produce any alterations in the muscular CAT, SOD2 and total antioxidant capacity (TAC) levels in the skeletal muscles irrespective of presented metabolism. However, chronic CBD exposure of the rats subjected to an HFD reduced the CAT level only in the red gastrocnemius muscle and enhanced the TAC in the white skeletal muscle. On the other hand, CBD administration notably increased the level of SOD2 in skeletal muscles with a different fiber type composition in the rats fed either a standard or an HFD. This was likely due to the fact that CBD was found to increase the mRNA level of SOD2 [24]; hence, we can assume that one of the mechanisms responsible for this CBD's action is Nrf2 signaling pathway activation. Additionally, it should be remarked that CBD has the ability to reduce the oxidative modifications of lipids that result from oxidation of membrane PUFAs by ROS [63]. This reaction generates unsaturated aldehydes, including malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are highly reactive and can lead to cellular dysfunctions. Our results, as expected, confirmed that rats in the state of HFD-induced obesity exhibited considerably elevated concentrations of both MDA and 4-HNE in the white and red gastrocnemius muscles. As regards the involvement of the muscle type in the effect of HFD feeding, the white gastrocnemius muscle demonstrated much higher concentrations of the abovementioned lipid peroxidation byproducts. This is probably caused by the structure of the white skeletal muscle which consists mainly of glycolytic fibers, using FA as an energy substrate to a small extent [61]. Most importantly, in the conducted experiment, we reported that treatment of the HFD-fed rats with CBD prevents the accumulation of MDA in both fast- and slow-twitch skeletal muscles. Furthermore, the 4-HNE concentration was decreased after prolonged CBD administration in the rats fed a standard chow or HFD in the white gastrocnemius muscle; however, these changes reached statistical significance only in the group of rats fed a standard diet. In addition, in the following study, we also evaluated the impact of CBD on advanced glycation end products (AGE) in conditions of chronic exposure to a high-fat diet. Not surprisingly, we reported that AGEs in rats on an HFD increased substantially in both muscle types, wherein the red gastrocnemius muscle exhibited intensified accumulation of AGEs in comparison with the white gastrocnemius muscle, which is consistent with results of other researchers [64]. This

may be due to basal metabolism and the muscle's adaptive response to overabundance of lipids during chronic exposure to an HFD. Noteworthily, two-week CBD administration to the high-fat diet fed animals caused a considerable reduction in the AGE concentration in both oxidative and glycolytic muscles. The mechanism of this effect is still not entirely clear but seems to involve multiple molecular pathways. Based on these findings, CBD could be a therapeutically useful agent in combating the effects of oxidative stress and inflammation associated with obesity and its metabolic disturbances.

5. Conclusions

Taken altogether, our results clearly demonstrated that an HFD promotes excessive accumulation of fatty acids in the skeletal muscle with a concomitant shift in the n-6/n-3 PUFA balance towards n-6 PUFAs, which is related to an increasing inflammatory response and oxidative stress and may contribute to obesity-associated onset of insulin resistance. Moreover, our data suggest that the impact of oxidative stress affects skeletal muscle function and metabolism when lipid FA overabundance is fiber type-specific. The major finding of the present study is that two-week CBD treatment effectively reduced the accumulation of fatty acids in muscular lipid pools and shifted the equilibrium of n-6/n-3 PUFAs in favor of anti-inflammatory n-3 PUFAs regardless of muscle metabolism. Moreover, CBD prevented generation of lipid peroxidation products, particularly in the glycolytic fibers. Concomitantly, it increased the antioxidative capacity of the muscles. Collectively, our observations emphasize the notion that chronic CBD administration may have a great therapeutic potential in the treatment of obesity-associated complications by alleviating inflammation and related lipid mediators as well as oxidative stress, which coincide with insulin resistance in obesity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nu13051603/s1, Supplementary File S1: The images of whole gels showing the total protein loading and the expression of selected proteins in the white and red gastrocnemius muscles in the control (standard diet) and high-fat diet (HFD) groups.

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11. Summary in polish

Mięśnie szkieletowe stanowią ważny organ zaangażowany w regulację licznych procesów metabolicznych organizmu poprzez wykorzystanie podstawowych substratów energetycznych takich jak glukoza oraz długołańcuchowe kwasy tłuszczowe (LCFA). Stopień utylizacji glukozy lub LCFA w warunkach fizjologicznych przez tkankę mięśniową zależy od wielu czynników, a w tym od dostępności i dokomórkowego transportu wyżej wymienionych substratów, zapotrzebowania energetycznego oraz równowagi hormonalnej organizmu. W warunkach zwiększonej dostępności kwasów tłuszczowych (FAs) w diecie, przewyższającej zapotrzebowanie energetyczne organizmu oraz zdolności oksydacyjne mitochondriów, dochodzi do magazynowania lipidów w tkance tłuszczowej, jak również w innych aktywnych metabolicznie tkankach takich jak mięśnie szkieletowe. Nadmierna wewnątrzmięśniowa akumulacja lipidów obserwowana jest głównie we frakcjach triacylogliceroli (TAG), diacylogliceroli (DAG) oraz ceramidów (CER). Liczne badania wykazały, że zwiększone stężenie bioaktywnych frakcji DAG i CER przyczynia się do upośledzenia działania insuliny co skutkuje rozwojem insulinooporności (IR) w tkance mięśniowej.

Liczne dane literaturowe wskazują, iż podczas rozwoju otyłości dochodzi do zwiększonej aktywacji wybranych komponentów układu endokannabinoidowego (ECS). Układ ten zaangażowany jest w regulację wielu procesów fizjologicznych, między innymi metabolizmu oraz homeostazy energetycznej organizmu. Wykazano, że ECS stanowi również jedno z miejsc docelowych oddziaływania fitokannabinoidów, będących związkami pochodzenia roślinnego (*Cannabis sativa*). Do tej grupy związków zaliczany jest kannabidiol (CBD), który ze względu na brak właściwości psychoaktywnych oraz dobry profil bezpieczeństwa stanowi potencjalnie terapeutyczny związek. Wiele badań wykazało korzystne działanie CBD, m.in. przeciwdrgawkowe, przeciwpsychotyczne, przeciwbólowe, przeciwzapalne oraz antyoksydacyjne. Postuluje się również, iż CBD może wywoływać korzystne efekty w leczeniu otyłości, pomimo tego, iż większość mechanizmów jego działania nie jest obecnie poznana. W związku z powyższym, celem przeprowadzonych badań było określenie wpływu CBD na metabolizm lipidów w mięśniach szkieletowych w szczurzym modelu otyłości indukowanej dietą bogatotłuszczową.

Badania zostały przeprowadzone na samcach szczurów rasy Wistar, które były karmione standardową karmą lub dietą wysokotłuszczową (HFD) przez 7 tygodni; każda grupa eksperymentalna składała się z 10 osobników. Zwierzęta, począwszy od szóstego tygodnia,

otrzymywały raz dziennie dootrzewnowe iniekcje z CBD w dawce 10 mg/kg masy ciała lub jego rozpuszczalnika przez kolejne 14 dni trwania eksperymentu. W uzyskanym do analiz materiale mięśni szkieletowych określone zostało stężenie wybranych frakcji lipidowych i sfingolipidów oraz zawartość poszczególnych kwasów tłuszczowych w badanych frakcjach przy użyciu chromatografii gazowo-cieczowej (GLC) oraz wysokosprawnej chromatografii cieczowej (HPLC). Osoczowe stężenia glukozy oraz insuliny, wewnątrzmięśniowa zawartość glikogenu oraz parametry stresu oksydacyjnego zostały oznaczone z użyciem metod kolorymetrycznych oraz immunoenzymatycznych (ELISA). Dodatkowo, techniką Western Blot oceniono ekspresję wybranych białek. Dane analizowano za pomocą jednokierunkowej ANOVA, a następnie odpowiedniego testu post-hoc ($p < 0.05$ poziom istotności).

Wyniki badań wskazują, że w mięśniach szkieletowych szczurów karmionych dietą bogatotłuszczową dochodzi do wzmożonej akumulacji niektórych frakcji lipidowych (DAG, TAG) oraz sfingolipidowych (CER), co związane jest z pogorszeniem przekaźnictwa sygnału insuliny. Jednocześnie dochodzi do zwiększenia stosunku n-6/n-3 PUFA, skutkiem czego jest zwiększona odpowiedź zapalna w tkance mięśni szkieletowych. Obiecujące wydają się efekty działania CBD, które wskazują na zmniejszenie zawartości wyżej wymienionych lipidów, obniżenie stosunku n-6/n-3 PUFA poprzez przesunięcie równowagi na korzyść przeciwzapalnych kwasów tłuszczowych n-3 PUFA, jak również poprawę transdukcji sygnału insuliny w mięśniach szkieletowych w warunkach indukowanej dietą bogatotłuszczową otyłości.

Otrzymane przez nas wyniki badań dostarczają nowych informacji o roli CBD jako regulatora metabolizmu lipidów w mięśniach szkieletowych i wskazują, że CBD ma potencjalne właściwości terapeutyczne w leczeniu otyłości i związanych z nią zaburzeń.

12. Summary in English

Skeletal muscles are an important organ involved in the regulation of numerous metabolic processes in the body through the use of basic energy substrates such as glucose and long-chain fatty acids (LCFA). The degree of glucose or LCFA utilization under physiological conditions by the muscle tissue depends on many factors, including the availability and intracellular transport of energy substrates, energy requirements, and the hormonal balance of the body. In conditions of increased availability of fatty acids (FAs) in the diet, exceeding the body's energy requirements and the oxidative capacity of the mitochondria, lipids are stored in adipose tissue as well as in other metabolically active tissues such as skeletal muscles. Excessive intramuscular lipid accumulation is mainly observed in the triacylglycerol (TAG), diacylglycerol (DAG) and ceramide (CER) lipid fractions. Numerous studies have shown that the increased concentration of bioactive DAG and CER fractions contributes to the impairment of insulin action, which results in the development of insulin resistance (IR) in muscle tissue.

Numerous literature data show that during the course of obesity, increased activation of selected components of the endocannabinoid system (ECS) is observed. This system is involved in the regulation of numerous physiological processes, including the metabolism and energy homeostasis of the organism. It has been shown that the ECS is also one of the target sites of the phytocannabinoid interactions, which are compounds of plant origin (Cannabis sativa). This group of compounds includes cannabidiol (CBD), which, due to its lack of psychoactive properties and good safety profile, is a potential therapeutic compound. Many studies have shown the positive effects of CBD, including anticonvulsant, antipsychotic, analgesic, and anti-inflammatory actions. It is also postulated that CBD may have beneficial effects in the treatment of obesity, despite the fact that most of its mechanisms of action are currently unknown. Therefore, the aim of the study was to determine the effect of CBD on lipid metabolism in skeletal muscle in a rat model of obesity induced by a high-fat diet.

All experiments were conducted on male Wistar rats, which were fed a standard diet or high-fat diet (HFD) for 7 weeks; each experimental group consisted of 10 individuals. From the beginning of the sixth week, the animals received once-daily intraperitoneal injections of CBD at a dose of 10 mg/kg of body mass or its solvent for the next 14 days of the experiment. In the material of skeletal muscle obtained for analysis, the concentration of selected lipid fractions and sphingolipids as well as the content of individual fatty acids in the examined fractions were

determined using gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). Plasma glucose and insulin levels, intramuscular glycogen content, and parameters of oxidative stress were determined using colorimetric and enzyme immunoassay (ELISA) methods. Additionally, the expression of selected proteins was assessed by the Western Blot technique. Data were analyzed by one-way ANOVA followed by an appropriate post-hoc test ($p < 0.05$ considered significant).

The results obtained in our research indicate that in the skeletal muscles of rats fed a high-fat diet there is an increased accumulation of examined lipid fractions (DAG, TAG) and CER, which is related to the deterioration of insulin signaling. Concomitantly, the n-6/n-3 PUFA ratio is elevated, resulting in an increased inflammatory response in skeletal muscle tissue. The effects of CBD seem highly promising, indicating a reduction in the above-mentioned lipids content, lowering the n-6/n-3 PUFA ratio by shifting the balance in favor of the anti-inflammatory n-3 PUFA, as well as improving insulin signal transduction in skeletal muscles under the conditions of high-fat diet-induced obesity.

Our data provide a new insight into the role of CBD as a regulator of lipid metabolism in skeletal muscle and indicate that CBD presents potential therapeutic properties with respect to the treatment of obesity and related disturbances.

13. Co-author's statements

Phytocannabinoids: useful drugs for the treatment of obesity? Special focus on cannabidiol.		
Author's name and surname	Nature of participation	Contribution in %
mgr Patrycja Bielawiec	Conception and design, literature collection and analysis, visualization, and manuscript preparation.	80%
dr n. med. Ewa Harasim-Symbor	Conception and design, reviewing and editing a manuscript.	10%
prof. Adrian Chabowski	Supervision, reviewing and editing a manuscript.	10%

Chronic cannabidiol administration attenuates skeletal muscle de novo ceramide synthesis pathway and related metabolic effects in a rat model of high-fat diet-induced obesity.		
Author's name and surname	Nature of participation	Contribution in %
mgr Patrycja Bielawiec	Conception and design, methodology, bioinformatics analysis and interpretation of the data, visualization and manuscript preparation.	70%
dr n. med. Ewa Harasim-Symbor	Conception and design, bioinformatics analysis and interpretation of the data, reviewing and editing a manuscript.	10%
dr n. med Karolina Konstantynowicz-Nowicka	Methodology and bioinformatics analysis of the data.	5%
mgr Klaudia Sztolsztener	Methodology and bioinformatics analysis of the data.	5%
prof. Adrian Chabowski	Supervision, reviewing and editing a manuscript.	10%

Attenuation of oxidative stress and inflammatory response by chronic cannabidiol administration is associated with improved n-6/n-3 PUFA ratio in the white and red skeletal muscle in a rat model of high-fat diet-induced obesity.		
Author's name and surname	Nature of participation	Contribution in %
mgr Patrycja Bielawiec	Conception and design, methodology, bioinformatics analysis and interpretation of the data, visualization and manuscript preparation.	70%
dr n. med. Ewa Harasim-Symbor	Conception and design, bioinformatics analysis and interpretation of the data, reviewing and editing a manuscript	10%
mgr Klaudia Sztolsztener	Methodology and bioinformatics analysis of the data.	5%
dr n. med Karolina Konstantynowicz-Nowicka	Methodology and bioinformatics analysis of the data.	5%
prof. Adrian Chabowski	Supervision, reviewing and editing a manuscript.	10%

I hereby declare that that all co-authors agreed to use these manuscripts in the dissertation of Patrycja Bielawiec.

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I agree to use this publication by Patrycja Bielawiec, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

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