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Dziedzina: nauki medyczne i nauki o zdrowiu

Dyscyplina: nauki medyczne

ROZPRAWA DOKTORSKA

Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną

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Spis treści

Rozdział 1. Wykaz publikacji będących podstawą rozprawy doktorskiej

1.1. Praca przeglądowa

1. Krzyżewska A., Baranowska-Kuczko M., Mińczuk K., Kozłowska H.: Cannabinoids-A New Perspective in Adjuvant Therapy for Pulmonary Hypertension. Int J Mol Sci., 2021, 22:10048.

Punktacja IF: **6.208**; MNiSW: **140**

1.2. Prace oryginalne

1. Krzyżewska A., Baranowska-Kuczko M., Jastrząb A., Kasacka I., Kozłowska H.: Cannabidiol Improves Antioxidant Capacity and Reduces Inflammation in the Lungs of Rats with Monocrotaline-Induced Pulmonary Hypertension. Molecules., 2022, 27:3327.

Punktacja IF: **4.600**; MNiSW: **140**

2. Krzyżewska A., Baranowska-Kuczko M., Kasacka I., Kozłowska H.: Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats. Biochim Biophys Acta Mol Basis Dis., 2023, 1869:166753.

Punktacja IF: **6.200**; MNiSW: **140**

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Rozdział 2. Wykaz stosowanych skrótów

2-AG	*(2-arachidonoylglycerol)* 2-arachidonyloglicerol
2-AGE	*(noladin ether)* eter noladyny
4-HNE	*(4-hydroxynonenal)* 4-hydroksynonenal
α-SMA	*(alpha-smooth muscle actin)* alfa-aktyna mięśni gładkich
Abn-CBD	*(abnormal cannabidiol)* atypowy kannabidiol
AEA	*(N-arachidonoylethanolamine)* *N*-arachidonoylethanolamina, anandamid
BMPR2	*(bone morphogenetic protein type 2 receptor)* receptor białka morfogenetycznego kości typu 2
CB_1/CB_2	*(cannabinoid receptors type 1 and 2)* receptory kannabinoidowe typu 1 i 2
CBD	*(cannabidiol)* kannabidiol
CD68	*(cluster of differentiation 68)* antygen różnicowania komórkowego 68
cGMP	*(cyclic guanosine monophosphate)* cykliczny guanozynomonofosforan
COX	*(cyclooxygenase)* cyklooksygenaza
COX-2	*(cyclooxygenase-2)* cyklooksygenaza-2
CTR	*(control group)* grupa kontrolna
eCB	*(putative endothelial cannabinoid receptor)* śródbłonkowy receptor kannabinoidowy
ECS	*(endocannabinoid system)* układ endokannabinoidowy
EndoMT	*(endothelial-to-mesenchymal transition)* przejście endotelialno-mezenchymalne
eNOS	*(endothelial nitric oxide synthase)* śródbłonkowa syntaza tlenku azotu
EP_4	*(prostaglandin E2 receptor 4)* receptor prostaglandynowy EP4
ERS	*(European Respiratory Society)* Europejskie Towarzystwo Oddechowe
ESC	*(European Society of Cardiology)* Europejskie Towarzystwo Kardiologiczne
ET-1	*(endothelin-1)* endotelina-1

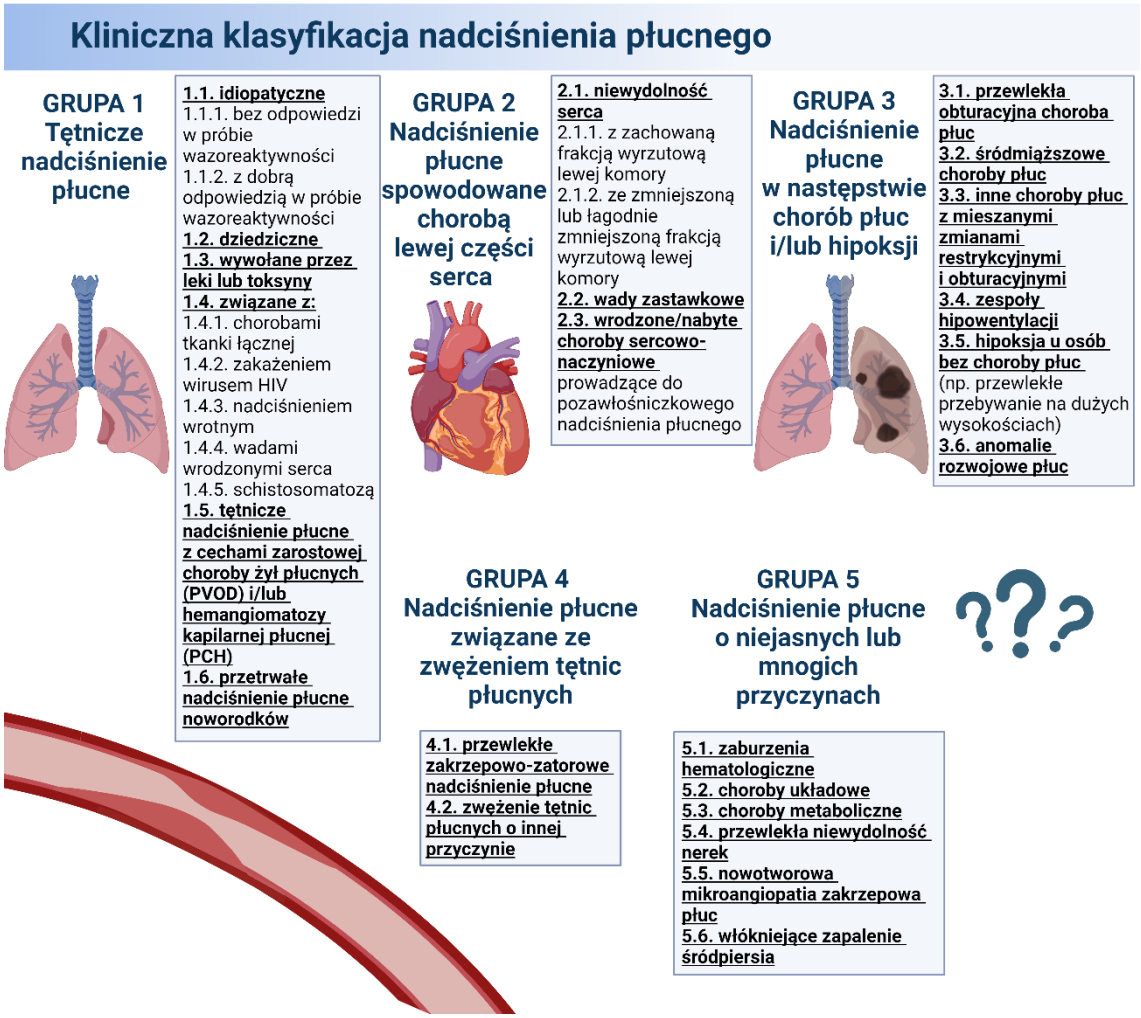
FAAH	*(fatty-acid amide hydrolase)* hydrolaza amidowa kwasów tłuszczowych
FGF2	*(fibroblast growth factor 2)* czynnik wzrostu fibroblastów 2
Gal-3	*(galectin-3)* galektyna-3
GPR18 / GPR55	*(G protein-coupled receptor 18, 55)* sierocy receptor sprzężony z białkiem G18 / G55
GSH	*(gluthatione)* glutation
GSR	*(glutathione reductase)* reduktaza glutationowa
IL-1β	*(interleukin 1 beta)* interleukina 1 beta
IL-6	*(interleukin 6)* interleukina 6
i.p.	*(intraperitoneally)* podanie dootrzewnowe
IP	*(prostacyclin receptor)* receptor prostacyklinowy
LPI	*(lysophosphatidylinositol)* L-alfa-lizofosfatydyloinozytol
LPS	*(lipopolysaccharide)* lipopolisacharyd
MAGL	*(monoacylglycerol lipase)* lipaza monoacyloglicerolowa
MCP-1	*(monocyte chemoattractant protein-1)* białko chemotaktyczne monocytów-1
MCT	*(monocrotaline)* monokrotalina
MMP-9	*(matrix metalloproteinase-9)* metaloproteinaza macierzy-9
NF-κB	*(nuclear factor kappa B)* jądrowy czynnik transkrypcyjny kappa B
NO	*(nitric oxide)* tlenek azotu
NT-proBNP	*(N-terminal pro b-type natriuretic peptide)* peptyd natriuretyczny typu pro-B
PAH	*(pulmonary arterial hypertension)* tętnicze nadciśnienie płucne
PAI-1	*(plasminogen activator inhibitor-1)* inhibitor aktywatora plazminogenu-1
pEC_{50}	ujemny logarytm z molowego stężenia agonisty powodującego połowę efektu (skurczu/rozkurczu) maksymalnego
PGI_2	*(prostacyclin)* prostacyklina
PH	*(pulmonary hypertension)* nadciśnienie płucne
PPAR-γ	*(peroxisome proliferator-activated receptor gamma)* receptor aktywowany przez proliferatory peroksysomów gamma
ROS	*(reactive oxygen species)* reaktywne formy tlenu

RVSP	*(right ventricular systolic pressure)* skurczowe ciśnienie w prawej komorze serca
s.c.	*(subcutaneously)* podanie podskórne
sGC	*(soluble guanylyl cyclase)* rozpuszczalna cyklaza guanylanowa
TAC	*(total antioxidant capacity)* całkowita pojemność antyoksydacyjna
TGF-β	*(transforming growth factor beta)* transformujący czynnik wzrostu beta
THC	*(tetrahydrocannabinol)* Δ^9-tetrahydrokannabinol
TNF-α	*(tumor necrosis factor alfa)* czynnik martwicy nowotworu alfa
t-PA	*(tissue-type plasminogen activator)* tkankowy aktywator plazminogenu
TRPV1	*(transient receptor potential cation channel subfamily V member 1)* receptor waniloidowy przejściowego potencjału 1
TXA_2	*(thromboxane A_2)* tromboksan A_2
VEGF	*(vascular endothelial growth factor)* czynnik wzrostu śródbłonka naczyniowego
VE-kadheryna	*(vascular endothelial cadherin)* kadheryna śródbłonka naczyniowego

Rozdział 3. Wprowadzenie

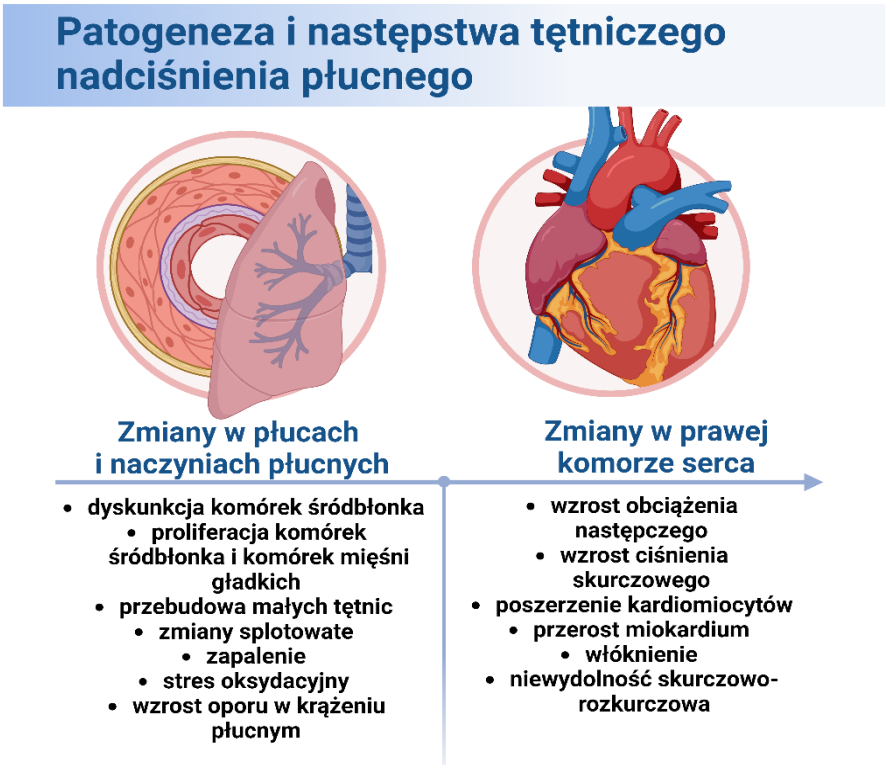
3.1. Nadciśnienie płucne

Nadciśnienie płucne (ang. *pulmonary hypertension* – PH) jest nieuleczalną, postępującą chorobą o złym rokowaniu. Szacuje się, że problem PH dotyka około 1% światowej populacji. Zgodnie z najnowszymi wytycznymi PH definiuje się średnim ciśnieniem w tętnicy płucnej w spoczynku wynoszącym > 20 mmHg (Humbert i wsp. 2022). Aktualną klasyfikację PH opracowaną w oparciu o podobne mechanizmy patofizjologiczne, obraz kliniczny oraz postępowanie terapeutyczne przedstawiono na Rycinie 1.



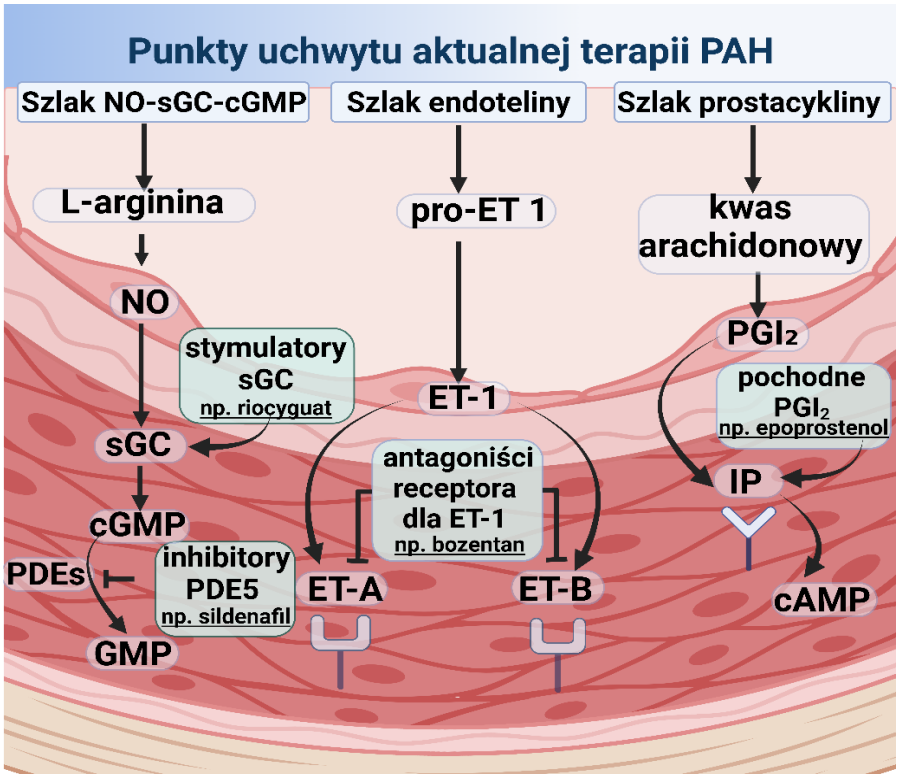
Rycina 1. Kliniczna klasyfikacja nadciśnienia płucnego. Przygotowano na podstawie Humbert i wsp. (2022). Stworzone w Biorender.com.

Tętnicze nadciśnienie płucne (ang. *pulmonary arterial hypertension* - PAH) należy do grupy 1 i jest jednym z najrzadziej występujących typów PH. Patogeneza PAH jest wieloczynnikowa i obejmuje: 1/ dysfunkcję i apoptozę komórek śródbłonka tętnic płucnych, 2/ nadmierną proliferację komórek śródbłonka oraz komórek mięśni gładkich tętnic płucnych, 3/ zapalenie i stres oksydacyjny, oraz 4/ miejscowe zmiany zakrzepowe w naczyniach płucnych. Dysfunkcyjny śródbłonek wydziela większe ilości substancji o działaniu naczyniozwężającym tj.: tromboksan A_2 (TXA_2), endotelina-1 (ET-1) oraz czynniki proliferacyjne takie jak: czynnik wzrostu śródbłonka naczyniowego (VEGF), czynnik wzrostu fibroblastów 2 (FGF2), a wydzielanie substancji o charakterze naczyniorozszerzającym, np.: tlenek azotu (NO) czy prostacyklina (PGI_2) zostaje zmniejszone (Kurakula i wsp. 2021). Wspominane zmiany manifestują się przebudową dystalnych tętniczek płucnych (o średnicy 20-500 μm) z wytworzeniem charakterystycznych zmian splotowatych (małe kanały kapilarne zlokalizowane wewnątrz ścian pogrubiałych tętniczek, tworzące wrażenie splotu naczyniowego), co powoduje wzrost oporu naczyniowego w krążeniu płucnym i w konsekwencji prowadzi do nadmiernego obciążenia następczego prawej komory serca. Obciążona komora ulega przebudowie, przerostowi i włóknieniu, co skutkuje pogorszeniem jej funkcji skurczowo-rozkurczowej i niewydolnością, która wiąże się z gorszym rokowaniem i przedwczesną śmiercią pacjentów (Andersen i wsp. 2019; Hassoun, 2021). Na Rycinie 2 została przedstawiona patogeneza i następstwa PAH.



Rycina 2. Patogeneza i następstwa tętniczego nadciśnienia płucnego. Stworzone w Biorender.com.

Aktualna terapia PAH skupia się przede wszystkim na działaniu naczyniorozszerzającym i jest ukierunkowana na modyfikacje szlaków NO - rozpuszczalnej cyklazy guanylanowej (sGC) - cyklicznego guanozynomonofosforanu (cGMP), endoteliny-1 i prostacykliny (Rycina 3) (Humbert i wsp. 2022). Taka strategia terapeutyczna spowalnia postęp choroby i poprawia jakość życia pacjentów, jednakże wciąż nie we wszystkich przypadkach osiągnięte zostają indywidualne cele lecznicze, a ogólne rokowanie pozostaje niekorzystne. Podkreśla to pilną potrzebę opracowania nowych strategii terapeutycznych o działaniu przyczynowym (Humbert i wsp. 2022). Obecnie do najbardziej obiecujących i cieszących się dużym zainteresowaniem rozwiązań terapeutycznych należą: inhibitory sygnalizacji aktywiny (terapie ukierunkowane na szlak transformującego czynnika wzrostu beta (ang. *transforming growth factor beta* - TGF-β)), inhibitory kinazy tyrozynowej (wykorzystują działanie antyproliferacyjne), a także podejście przeciwzapalne, które wydaje się być korzystnym rozwiązaniem dla pacjentów z PAH związanym z chorobami tkanki łącznej, które często mają podłoże autoimmunologiczne (Humbert i wsp. 2023a).



Rycina 3. Punkty uchwytu aktualnej terapii tętniczego nadciśnienia płucnego wraz z wybranymi przykładami leków. Opracowano na podstawie Humbert i wsp. (2023b). Skróty: cAMP: cykliczny adenozynomonofosforan; cGMP: cykliczny guanozynomonofosforan; ET-1: endotelina-1; ET-A/ET-B: receptor dla endoteliny typu A/B; IP: receptor dla prostacykliny; PDEs: fosfodiesterazy; PDE5: fosfodiesteraza typu 5; PGI_2: prostacyklina; pro-ET1: proendotelina 1; NO: tlenek azotu; sGC: rozpuszczalna cyklaza guanylanowa. Stworzone w Biorender.com.

3.2. Rola oraz interakcja szlaków sygnałowych transformującego czynnika wzrostu beta i jądrowego czynnika transkrypcyjnego kappa B w inicjacji i rozwoju tętniczego nadciśnienia płucnego

Pomimo że jednymi z cech charakterystycznych PAH są sztywność tętniczek płucnych i reakcje zapalne, to wciąż wiedza na temat roli zapalenia i dokładnych szlaków molekularnych zaangażowanych w inicjację przebudowy naczyń płucnych i prawej komory serca wydaje się ograniczona. Dowiedziono, że stan zapalny promuje proliferację komórek mięśni gładkich naczyń i odkładanie się macierzy zewnątrzkomórkowej, co skutkuje pogrubieniem, zmniejszeniem podatności i przebudową ścian naczyń krwionośnych w PAH (Liu i wsp. 2022). Wiadomo, że jądrowy czynnik transkrypcyjny kappa B (ang. *nuclear factor kappa B* - NF-κB) jest centralnym regulatorem stanu zapalnego, a jego aktywacja zwiększa ekspresję cytokin prozapalnych takich jak interleukina 1 beta (IL-1β), interleukina 6 (IL-6) czy czynnik martwicy nowotworu alfa (TNF-α), które bezpośrednio kontrolują szereg procesów zachodzących w komórkach

naczyń płucnych w tym proliferację, migrację i różnicowanie (Rabinovitch i wsp. 2014). Pomimo że badania nad rolą NF-κB w rozwoju PAH są na wstępnym etapie, dowiedziono już, że pacjenci z idiopatycznym PAH (iPAH) wykazują zwiększoną aktywację tego czynnika w obrębie zmian splotowatych, a podobne obserwacje zauważono także w tętnicach płucnych szczurów w modelu eksperymentalnym PH indukowanym przewlekłym niedotlenieniem i Sugenem 5416 (inhibitor receptora czynnika wzrostu śródbłonka naczyniowego) (Farkas i wsp. 2014).

W badaniach nad patologicznymi zmianami zachodzącymi zarówno w naczyniach płucnych jak i w prawej komorze serca w przebiegu PAH coraz większą uwagę poświęca się przejściu endotelialno-mezenchymalnemu (EndoMT), które definiuje się jako proces, w którym komórki śródbłonka przechodzą transformację w komórki mezenchymalne. Zjawisko EndoMT istotnie zaburza homeostazę prawidłowego śródbłonka prowadząc do jego dysfunkcji i zmieniając jego fenotyp na proliferacyjny (Gorelova i wsp. 2021). Szczególna rola EndoMT w dysfunkcji prawej komory serca została opisana szerzej w pracy oryginalnej stanowiącej część tej rozprawy i znajduje się w Rozdziale 12. Wykazano, iż w inicjacji EndoMT oprócz już dobrze udokumentowanej utraty funkcji receptora białka morfogenetycznego kości typu 2 (ang. *bone morphogenetic protein type 2 receptor* - BMPR2) (kodowanego przez gen *BMPR2*, którego mutacja jest najczęstszą genetyczną przyczyną PAH), odgrywają rolę także nadekspresja czynników transkrypcyjnych (m.in. NF-κB,) oraz cytokin prozapalnych (TNF-α, IL-1β, IL-6) poprzez aktywację sygnalizacji TGF-β (Thenappan i wsp. 2018). Wydaje się zatem, że dwie cząsteczki sygnałowe tj. TGF-β i NF-κB odpowiedzialne pośrednio i bezpośrednio zarówno za rozwój stanu zapalnego jak i proliferację oraz przebudowę mogą być obiecującym celem przyszłych terapii PAH. Ponadto nadrodzina TGF-β obejmuje szereg białek funkcyjnych (w tym TGF-β1, aktywina), których aktywacja skutkuje odpowiedzią ze strony niekanonicznych szlaków sygnałowych i szlaku kanonicznego (SMAD) (Guignabert i wsp. 2021). Wiadomo, że zaburzenie równowagi pomiędzy elementami nadrodziny TGF-β jest obecnie uważane za główny defekt molekularny odgrywający kluczową rolę w predyspozycji do rozwoju PAH, co skutkuje zmianą fenotypu śródbłonka na proliferacyjny i postępie choroby. Niedawne badania nad nowym białkiem fuzyjnym – sotaterceptem ukierunkowanym na aktywinę i innych członków nadrodziny TGF-β są aktualnie postrzegane jako długo wyczekiwany przełom w terapii PAH (Humbert i wsp. 2021; Humbert 2023b; Humbert i wsp. 2023c; Kopeć 2023).

3.3. Eksperymentalne modele nadciśnienia płucnego - model monokrotalinowy

Ze względu na heterogenny charakter PH, nie opracowano jeszcze doskonałego przedklinicznego modelu odwzorowującego ludzkie PAH, jednakże modele zwierzęce dostarczyły wartościowych wniosków dotyczących rozwoju tego schorzenia. Wybór odpowiedniego modelu do badania PAH ma kluczowe znaczenie, nie tylko w badaniach nad mechanizmami patofizjologicznymi choroby, ale również w badaniach nowych, potencjalnych strategii terapeutycznych. Aktualnie dysponujemy zarówno klasycznymi, jak i nowatorskimi sposobami modelowania, do których zaliczamy: 1/ modele bezinwazyjne *in vivo* (np.: przewlekłe niedotlenienie, przewlekłe niedotlenienie z podaniem Sugenu 5416, model z podaniem monokrotaliny (MCT)), 2/ modele inwazyjne *in vivo* (np.: pneumonektomia), 3/ modele genetyczne (np.: mutacja genu *BMPR2*), 4/ PH indukowane wieloma czynnikami np.: monokrotalina/pneumonektomia, model Sugen 5416/pneumonektomia. Obecnie dwoma najpowszechniej stosowanymi modelami są model monokrotalinowy oraz model Sugen 5416/hipoksja (Wu i wsp. 2022; Dignam i wsp. 2022).

Badania wchodzące w skład rozprawy dotyczą PAH (grupa 1), jednak ze względu na fakt, że eksperymentalne modele zwierzęce różnią się od przebiegu ludzkiego PAH (szczególnie dotyczy to braku rozwoju charakterystycznych zmian splotowatych w modelu monokrotalinowym), termin PAH w całej rozprawie i wchodzących w jej skład publikacjach odnosi się do warunków ludzkich, a termin doświadczalne PH do warunków eksperymentalnych (Hill i wsp. 2017).

Monokrotalina to alkaloid pirolizydynowy otrzymywany z nasion rośliny *Crotalaria spectabilis*. Indukcja modelu polega na pojedynczym, podskórnym podaniu MCT (50–80 mg/kg), która w organizmie ulega metabolizmowi do pirolu MCT, a ten prowadzi do uszkodzenia śródbłonka naczyniowego, zapalenia okołonaczyniowego i muskularyzacji małych tętniczek płucnych, co pozwala na rozwinięcie PH po około 3-4 tygodniach od ekspozycji. Preferowanym gatunkiem do badań jest szczur, ze względu na brak metabolizmu MCT do aktywnej formy u myszy. Model MCT pozwala również na rozwój przerostu prawej komory serca, a do innych zalet można zaliczyć także jego powtarzalność (Wu i wsp. 2022).

3.4. Kannabinoidy – kannabidiol

Kannabinoidy to grupa związków lipofilnych, które są ligandami receptorów kannabinoidowych (zarówno klasycznych tj. receptory kannabinoidowe typu 1 i 2 (CB_1, CB_2) jak i nieklasycznych np. sierocy receptor sprzężony z białkiem G55 (GPR55)). Obecna klasyfikacja kannabinoidów opiera się na ich pochodzeniu: 1/ fitokannabinoidy izolowane z *Cannabis sativa* np.: Δ^9-tetrahydrokannabinol (THC), kannabidiol (CBD); 2/ endokannabinoidy i cząsteczki podobne do endokannabinoidów, naturalnie występujące w organizmie ssaków np.: 2-arachidonyloglicerol (2-AG) i *N*-arachidonoylethanolamina (anandamid, AEA); oraz 3/ związki otrzymywane na drodze syntezy chemicznej np. WIN 55,212-2 (Maccarrone i wsp. 2023).

Kannabinoidy są wykorzystywane od wieków w celach rekreacyjnych i leczniczych, a coraz więcej dowodów sugeruje, że mogą wykazywać korzystny wpływ na układ oddechowy i krwionośny. Wiadomo już, że w niektórych typach nadciśnienia, w tym w PH, układ endokannabinoidowy (ECS) ulega zwiększonej regulacji, a jego składowe (np.: receptory CB_1, CB_2 i AEA) są obecne zarówno w naczyniach płucnych jak i tkance płucnej, co sugeruje, że mogą stanowić potencjalny punkt uchwytu nowych terapii PH i innych stanów klinicznych związanych z krążeniem płucnym i układem oddechowym (Kicman i wsp. 2021; Krzyżewska i wsp. 2021; Remiszewski i wsp. 2022). Kannabinoidy oprócz szeregu korzystnych właściwości w tym: działania przeciwzapalnego, antyoksydacyjnego i przeciwzwłóknieniowego wykazują także wielokierunkowe działanie rozszerzające naczynia systemowe oraz naczynia płucne, w którym pośredniczy śródbłonek naczyniowy i/lub szlak zależny od cyklooksygenaz (COX), kanałów potasowych aktywowanych jonami wapnia (K_{Ca}), receptorów kannabinoidowych i innych, co szerzej zostało opisane w pracy przeglądowej stanowiącej część mojej rozprawy doktorskiej (Rozdział 10). Jednym z najlepiej przebadanych kannabinoidów jest CBD, który nie wykazuje działania psychoaktywnego i posiada dobry profil bezpieczeństwa. Kannabidiol wchodzi w skład preparatu Epidiolex i został zatwierdzony przez Amerykańską Agencję Żywności i Leków w leczeniu padaczki lekoopornej. Kannabidiol wykazuje wielokierunkowe korzystne działanie m. in.: przeciwbólowe, przeciwdrgawkowe, przeciwastmatyczne, neuroprotekcyjne, przeciwlękowe, obniżające ciśnienie krwi indukowane stresem, a także, co wydaje się istotne w kontekście PAH, wykazuje działanie: rozkurczające naczynia płucne,

antyoksydacyjne, przeciwzapalne i przeciwzwłóknieniowe (Krzyżewska i wsp. 2021; Castillo-Arellano i wsp. 2023).

Kannabidiol zmniejszał ciśnienie skurczowe prawej komory serca (ang. *right ventricular systolic pressure* - RVSP) i przerost naczyń płucnych zarówno w szczurzym modelu PH wywołanym MCT (Sadowska i wsp. 2020; Lu i wsp. 2021), jak i w mysim modelu PH indukowanym podaniem Sugenu 5416 z jednoczesną ekspozycją na niedotlenienie (Lu i wsp. 2021), co sugeruje, że CBD może łagodzić objawy PH. Kannabidiol wykazuje także działanie rozszerzające ludzkie naczynia płucne (Baranowska-Kuczko i wsp. 2020), a chroniczne podawanie CBD (10 mg/kg przez 21 dni) szczurom z PH indukowanym MCT było skuteczne w poprawie relaksacji naczyń płucnych zależnej od śródbłonka w odpowiedzi na acetylocholinę (Sadowska i wsp. 2020).

Dodatkowo CBD reguluje równowagę oksydoredukcyjną, wychwytuje wolne rodniki tlenowe, przekształca je w mniej aktywne formy, a także zmniejsza wytwarzanie reaktywnych form tlenu (ROS) poprzez chelatowanie jonów metali przejściowych (Atalay i wsp. 2019). Kannabidiol zmniejsza stan zapalny w tkance płucnej w mysim modelu astmy (Vuolo i wsp. 2019) i w ostrym uszkodzeniu płuc wywołanym lipopolisacharydem (LPS) u myszy (Ribeiro i wsp. 2014). Wykazano także, że CBD hamuje szlak związany z NF-κB, który jest głównym regulatorem genów prozapalnych i tym samym zmniejsza ilość mediatorów zapalenia, między innymi IL-1β, IL-6 oraz TNF-α w komórkach mikrogleju BV-2 (Kozela i wsp. 2010).

Sugeruje się, że CBD może mieć wysoki potencjał kardioprotekcyjny. Przewlekłe podanie CBD miało korzystny wpływ na serca szczurów z nadciśnieniem pierwotnym i wtórnym zmniejszając szerokość kardiomiocytów w lewej komorze oraz redukując wywołany karbacholem skurcz naczyń wieńcowych (Pędzińska-Betiuk i wsp. 2021), jednak nie modyfikowało ciśnienia krwi (Remiszewski i wsp. 2020). Kannabidiol był również skuteczny w zmniejszaniu stresu oksydacyjnego i stanu zapalnego w szczurzym (Fouad i wsp. 2013) i mysim (Hao i wsp. 2015) modelu kardiomiopatii indukowanej doksorubicyną, a także ograniczał dysfunkcję i zwłóknienie miokardium w mysim autoimmunologicznym zapaleniu mięśnia sercowego (Lee i wsp. 2016).

Rozdział 4. Cel pracy z uzasadnieniem podjętej tematyki badawczej

Tętnicze nadciśnienie płucne to stan kliniczny, który charakteryzuje się wysokim oporem w krążeniu płucnym. Choroba ta ma wieloczynnikową etiologię szczególnie w naczyniach płucnych, która obejmuje dysfunkcję śródbłonka i ich nadmierną przebudowę, stan zapalny oraz stres oksydacyjny. W wyniku szeregu wspominanych zmian często dochodzi do wzrostu oporu w krążeniu płucnym, zwiększonego obciążenia następczego prawej komory serca i w konsekwencji jej przerostu oraz zmian zwłóknieniowych, które prowadzą do jej niewydolności i przedwczesnej śmierci pacjenta. Obecne strategie terapeutyczne zalecane przez Europejskie Towarzystwo Oddechowe (ERS) obejmują przede wszystkim leki o działaniu naczyniorozszerzającym, które przyczyniają się do poprawy stanu i jakości życia pacjenta, jednak nie pozwalają na pełne wyleczenie choroby. Sugeruje się, że najlepszą strategią terapeutyczną w leczeniu PAH jest wczesna politerapia celująca w kilka punktów uchwytu w tym: zmniejszenie oporu naczyniowego w krążeniu płucnym, przeciwdziałanie zmianom proliferacyjnym i zwłóknieniowym, działanie przeciwzapalne i antyoksydacyjne.

Szczurzy model PH indukowanego MCT pozwala na selektywne uszkodzenie naczyń płucnych i odzwierciedla większość cech ludzkiego PAH. Ponadto prostota wykonania i szerokie wykorzystanie w badaniach naukowych wspomnianego wyżej modelu pozwala na uzyskanie powtarzalnych rezultatów o wysokim potencjalne translacyjnym.

Kannabidiol to jeden z najlepiej przebadanych składników *Cannabis sativa*, który nie wykazuje działania psychoaktywnego i odznacza się wysokim profilem bezpieczeństwa. Do tej pory znalazł zastosowanie w leczeniu padaczki lekoopornej i spastyczności mięśni w przebiegu stwardnienia rozsianego. Kannabidiol ze względu na szeroki zakres korzystnych właściwości obejmujących działanie przeciwzapalne, antyoksydacyjne, przeciwzwłóknieniowe i rozszerzające naczynia płucne wydaje się być bardzo obiecującą propozycją terapeutyczną uzupełniającą istniejące już strategie lecznicze. Dodatkowo w badaniach eksperymentalnych PH, CBD obniżał skurczowe ciśnienie w prawej komorze serca i wykazywał działanie kardioprotekcyjne przy braku wpływu na ciśnienie systemowe, co czyni go substancją aktywnie czynną celującą w kilka punktów uchwytu patogenezy PAH.

W związku z powyższym, celem mojej rozprawy doktorskiej była:

1. analiza dostępnej literatury na temat wpływu kannabinoidów, w tym CBD, na krążenie płucne oraz ich ewentualnej przydatności w terapii PH,

2. ocena wpływu przewlekłego podawania CBD na parametry stresu oksydacyjnego, stanu zapalnego i poziom klasycznych receptorów kannabinoidowych CB_1 i CB_2 w płucach szczurów z PH indukowanym MCT,

3. ocena potencjału przeciwzwłóknieniowego CBD oraz zaangażowania w ten efekt szlaku sygnałowego TGF-β1/SMAD2 w prawej komorze serca szczurów z PH indukowanym MCT.

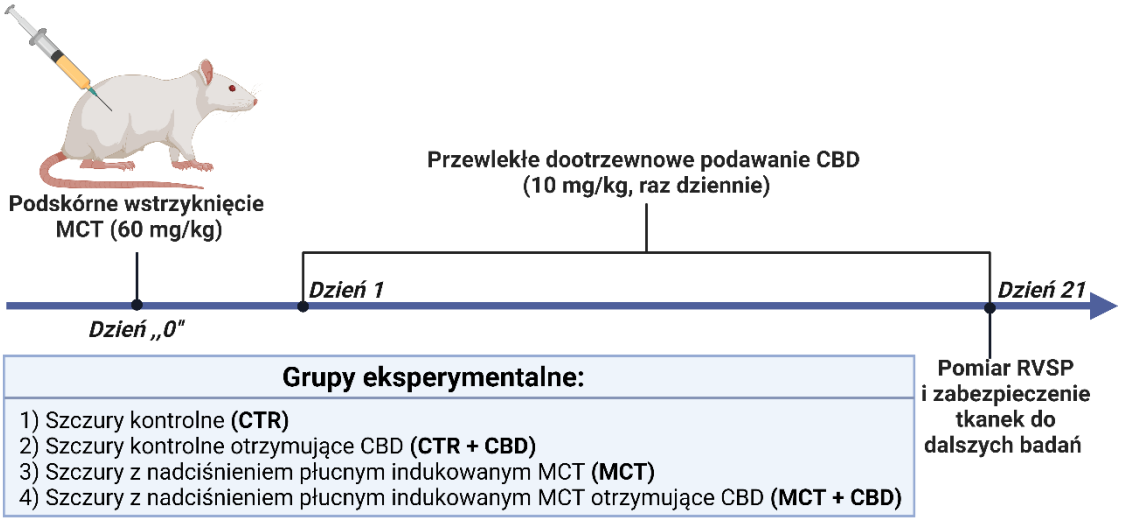
Rozdział 5. Realizacja celów naukowych, materiały i metody badawcze, podsumowanie wyników badań i dyskusja

5.1. Materiały i metody

Zgoda na wykonanie wszystkich procedur i doświadczeń została udzielona przez Lokalną Komisję Etyczną ds. Doświadczeń na Zwierzętach Uniwersytetu Warmińsko-Mazurskiego w Olsztynie (uchwała nr: 88/2018, zatwierdzony 27.11.2018). Doświadczenia wykonywano zgodnie z Dyrektywą Europejską (2010/63/EU) i wytycznymi ARRIVE (Percie du Sert i wsp. 2020). Przestrzegając zasady 3R tj. redukcja, zastąpienie i udoskonalenie (ang. *Reduction, Replacement, Refinement*) badania zostały wykonane na wcześniej pobranych i odpowiednio zabezpieczonych tkankach 40 szczurów Wistar płci męskiej (5-8 tygodniowych o wadze ok. 250 g), które miały wyindukowane PH za pomocą MCT. Moje obydwie prace oryginalne wchodzące w skład rozprawy doktorskiej stanowią kontynuację i uzupełnienie wyników pracy Sadowska i wsp. (2020).

5.2. Schemat doświadczeń

Zwierzęta podzielono losowo na 4 grupy. Szczurom wstrzyknięto MCT w objętości 3 ml/kg w dawce 60 mg/kg masy ciała jednorazowo podskórnie (*s.c.*) w dniu „0” lub rozpuszczalnik dla MCT w tej samej objętości. Zdrowym szczurom, jak również szczurom po wstrzyknięciu MCT podawano dootrzewnowo (*i.p.*) CBD (10 mg/kg) lub jego rozpuszczalnik co 24 godziny przez 21 dni. Otrzymano następujące 4 grupy eksperymentalne: (1) kontrola (CTR): szczury otrzymujące rozpuszczalnik MCT i rozpuszczalnik CBD; (2) CTR + CBD: szczury otrzymujące CBD i rozpuszczalnik MCT; (3) MCT: szczury otrzymujące MCT i rozpuszczalnik CBD; (4) MCT + CBD: szczury otrzymujące MCT i CBD. Dla uproszczenia grupy oznaczono w następujący sposób: CTR, CTR + CBD, MCT, MCT + CBD, z pominięciem w nazewnictwie użytych rozpuszczalników (Rycina 4).



Rycina 4. Protokół doświadczalny. CBD: kannabidiol; CTR: grupa kontrolna; *i.p.*: podanie dootrzewnowe; MCT: grupa szczurów z nadciśnieniem płucnym indukowanym monokrotaliną; RVSP: ciśnienie skurczowe w prawej komorze serca; *s.c.*: podanie podskórne. Stworzone w Biorender.com.

Tkanki, na których wykonywałam doświadczenia pochodziły od szczurów, które miały wyindukowane PH za pomocą MCT, co zostało potwierdzone następującymi zmianami patologicznymi (i zostało opublikowane wcześniej w pracy Sadowska i wsp. 2020):

- wzrost RVSP (dokładne wartości przedstawiono w Tabeli 1),
- przerost prawej komory serca,
- obrzęk płuc,
- przebudowa małych tętniczek płucnych,
- zmniejszenie wysycenia krwi tlenem.

Tabela 1. Wartości skurczowego ciśnienia w prawej komorze serca w każdej grupie.

Grupa	RVSP
CTR	20.03 ± 0.9 mmHg, n = 10
CTR + CBD	21.4 ± 0.9 mmHg, n = 10
MCT	43.7 ± 3.9 mmHg, n = 10, $p < 0.001$ względem grupy CTR
MCT + CBD	28.2 ± 0.7 mmHg, n = 10, $p < 0.001$ względem grupy MCT

Skróty: CBD: kannabidiol; CTR: grupa kontrolna; MCT: grupa szczurów z nadciśnieniem płucnym indukowanym monokrotaliną; n: liczba zwierząt; RVSP: skurczowe ciśnienie w prawej komorze.

Uzasadnienie zastosowanego modelu eksperymentalnego i doboru dawek zostały szczegółowo opisane w pracach oryginalnych zamieszczonych w Rozdziałach 11 i 12.

Metody, za pomocą których oznaczono parametry w tkankach płuc szczurów włączone do pierwszej pracy oryginalnej (Rozdział 11):

- Western Blot wykorzystano do oznaczeń: poziomu klasycznych receptorów kannabinoidowych - CB_1 i CB_2, antygenu różnicowania komórkowego 68 (CD68), cyklooksygenazy 2 (COX-2);
- Testy immunoenzymatyczne (ELISA)/kolorymetryczne wykorzystano do oznaczeń: stężenia białka chemotaktycznego monocytów-1 (MCP-1), IL-1β, TNF-α i NF-κB; całkowitą pojemność antyoksydacyjną (TAC) określono za pomocą komercyjnego zestawu wykorzystującego metodę kolorymetryczną;
- Metody immunohistochemiczne wykorzystano do oznaczeń immunoekspresji IL-1β, CD68, CB_1 i CB_2;
- Metoda „Mize and Langdon" została użyta do oznaczeń aktywności reduktazy glutationowej (GSR);
- Metoda elektroforezy kapilarnej została wykorzystana do oznaczeń poziomu glutationu (GSH);
- Chromatografia gazowa sprzężona ze spektrometrią mas została wykorzystana do oznaczeń poziomu 4-hydroksynonenalu (4-HNE).

Metody, za pomocą których oznaczono parametry w osoczu i prawych komorach serca szczurów włączone do drugiej pracy oryginalnej (Rozdział 12):

- Westen blot wykorzystano do oceny poziomu galektyny-3 (Gal-3), metaloproteinazy macierzy-9 (MMP-9), TGF-β1, forsforylowanej formy SMAD2 (pSMAD2), SMAD2, kadheryny śródbłonka naczyniowego (VE-kadheryna), alfa-aktyny mięśni gładkich (α-SMA);
- Metody histologiczne i immunohistochemiczne użyto do oceny: szerokości kardiomiocytów (barwienie H + E), obszaru zwłóknienia śródmiąższowego i okołonaczyniowego (Barwienie Trichrome Massona), immunoekspresji fibronektyny;
- Test ELISA wykorzystano do określenia stężenia osoczowego peptydu natriuretycznego typu pro-B (ang. *N-terminal pro b-type natriuretic peptide* - NT-proBNP).

Szczegółowy opis zastosowanych metod wraz z analizą statystyczną znajduje się w pracach oryginalnych (Rozdział 11 i 12).

5.3. Podsumowanie wyników badań i dyskusja

5.3.1. Kannabinoidy jako środki o potencjale naczyniorozszerzającym

Cykl mojej rozprawy doktorskiej rozpoczyna się pracą przeglądową, której celem było dokonanie analizy dostępnej literatury na temat wpływu kannabinoidów na płucne naczynia krwionośne, a także skutków działania kannabinoidów zaobserwowanych dotychczas podczas badań *in vivo* i *in vitro* w krążeniu płucnym i potencjalnego wykorzystania kannabinoidów w terapii PH.

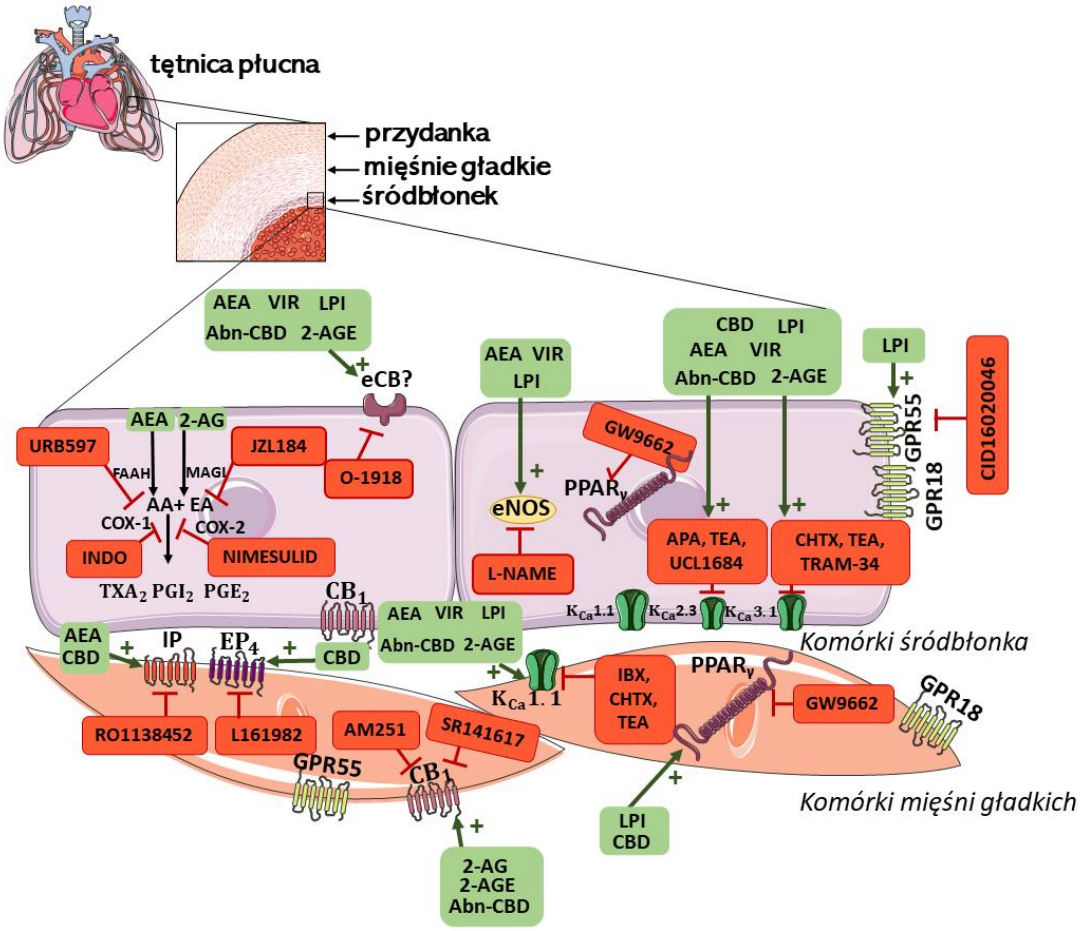
W krążeniu płucnym ludzi i zwierząt wykazano obecność ligandów endokannabinoidowych (m.in.: AEA, 2-AG), klasycznych (CB_1, CB_2) i nieklasycznych receptorów kannabinoidowych [receptor waniloidowy przejściowego potencjału 1 (TRPV1), sierocy receptor sprzężony z białkiem G18 (GPR18), czy G55 (GPR55)], a także enzymu odpowiedzialnego za degradację endokannabinodiów (hydrolaza amidowa kwasów tłuszczowych, FAAH), co potwierdza, iż kannabinoidy posiadają punkty działania w krążeniu płucnym (praca przeglądowa, Rozdział 10).

Kannabinoidy wykazują zależne od stężenia działanie rozszerzające ludzkie tętnice płucne. Zgodnie z wartością pEC_{50} (ujemny logarytm z molowego stężenia agonisty powodującego połowę efektu (skurczu/rozkurczu) maksymalnego) w ludzkich tętnicach płucnych, najsilniejsze działanie rozkurczowe posiada L-alfa-lizofosfatydyloinozytol (LPI) (pEC_{50} = 6,4) (Karpińska i wsp. 2018), z kolei 2-AG (Karpińska i wsp. 2017), AEA (w obecności antagonisty receptora CB_1 - AM251 (Kozłowska i wsp. 2007) oraz w badaniu bez udziału antagonisty (Baranowska-Kuczko i wsp. 2014)), wirodamina (VIR) (Kozłowska i wsp. 2008) i CBD mają podobną siłę działania (pEC_{50} = ~5), a atypowy kannabidiol (Abn-CBD) (Kozłowska i wsp. 2007) wykazywał najsłabszy efekt rozszerzający naczynia płucne (pEC_{50} = 4,8). Podobne wyniki uzyskano w naczyniach płucnych zwierząt (praca przeglądowa, Rozdział 10).

(Endo)kannabinoidy odgrywają rolę w regulacji napięcia naczyń płucnych poprzez mechanizmy zależne od śródbłonka i/lub oparte na receptorach, co może przyczyniać się do zmniejszenia oporu płucnego. We wszystkich badaniach przeprowadzonych na izolowanych tętnicach płucnych podsegmentarnych człowieka i szczura usunięcie śródbłonka osłabiało relaksację naczyń wywoływaną przez kannabinoidy (AEA, VIR, 2-AG, LPI, CBD, Abn-CBD, eter noladyny (2-AGE)). Sugeruje to udział mechanizmów zależnych od śródbłonka w tym zaangażowanie: 1/ śródbłonkowej syntazy tlenku azotu (eNOS) w relaksacji indukowanej ligandami

kannabinoidowymi AEA, VIR i LPI, 2/ pochodnych kwasu arachidonowego i enzymów uczestniczących w jego przemianie tj. FAAH w relaksacji indukowanej AEA i VIR; COX-1 i COX-2 w relaksacji indukowanej AEA, VIR CBD; i lipazy monoacyloglicerolowej (MAGL) w relaksacji indukowanej 2-AG, 3/ kanałów potasowych zależnych od wapnia w relaksacji indukowanej AEA, VIR, LPI, CBD, Abn-CBD. Oprócz powyższego część kannabinoidów wykazywała działanie rozszerzające naczynia płucne, w którym pośredniczyły receptory w tym: 1/ CB_1 dla 2-AG, 2-AGE, Abn-CBD, 2/ receptor prostacyklinowy (IP) dla AEA i CBD, 3/ receptor aktywowany przez proliferatory peroksysomów gamma (PPAR-γ) dla LPI i CBD, 4/ receptor prostaglandynowy EP4 (EP_4) dla CBD, 5/ TRPV1 dla CBD oraz 6/ śródbłonkowy receptor kannabinoidowy (eCB) dla AEA, VIR, LPI, Abn-CBD, 2-AGE (dokładne mechanizmy oraz działające przez nie ligandy kannabinoidowe opisano w pracy przeglądowej w Rozdziale 10 i przedstawiono na Rycinie 5).

W komentarzu redakcyjnym do publikacji Kozłowska i wsp. (2007), prof. Hornig (2007) postawił tezę, że kannabinoidy mogą stać się elementem terapii PAH, jednak wciąż mamy zbyt małą wiedzę na ten temat i potrzebne są dalsze eksperymenty. W związku z powyższym w mojej pracy przeglądowej przeanalizowałam przydatność kannabinoidów (ze względu na ograniczoną ilość dostępnej literatury, analiza skupiała się głównie na CBD) w badaniach *in vivo* w modelach zwierzęcych PH (PH indukowane MCT oraz PH indukowane Sugenem 5416 i przewlekłym niedotlenieniem) oraz w badaniach *in vitro* na ludzkich komórkach mięśni gładkich tętnicy płucnej eksponowanych na przewlekłe niedotlenienie. Wspomniane badania (z czego badania Sadowska i wsp. (2020) pochodzą z naszej pracowni badawczej) wykazały, że CBD skutecznie obniżał RVSP w szczurzym modelu PH indukowanym MCT, a także w mysim modelu PH indukowanego Sugenem 5416 i niedotlenieniem. Dodatkowo CBD był skuteczny w hamowaniu przerostu tętnic płucnych i prawej komory serca, zwiększał wysycenie krwi tlenem i stężenie endogennych kannabinoidów w tkance płucnej, a także normalizował wybrane parametry układu krzepnięcia (inhibitor aktywatora plazminogenu-1 (PAI-1) i tkankowy aktywator plazminogenu (t-PA) w osoczu w zwierzęcych modelach PH (Sadowska i wsp. 2020; Lu i wsp. 2021). Kannabidiol zapobiegał nadmiernej proliferacji, zmniejszał ekspresję mRNA wybranych chemokin oraz stres oksydacyjny w mitochondriach w ludzkich komórkach mięśni gładkich tętnicy płucnej w warunkach niedotlenienia (Lu i wsp. 2021).



Rycina 5. Lokalizacja elementów układu endokannabinoidowego i potencjalne mechanizmy zaangażowane w wywołany kannabinoidami rozkurcz tętnic płucnych. Skróty: 2-AG: 2-arachidonoiloglicerol; 2-AGE: eter noladyny; AA: kwas arachidonowy; Abn-CBD: atypowy kannabidiol; AEA: *N*-arachidonoylethanolamina/anandamid; AM251: antagonista receptora CB_1; AM630: antagonista receptora CB_2; APA: apamina, inhibitor $K_{Ca}2.3$; CB_1: receptor kannabinoidowy typu 1; CB_2: receptor kannabinoidowy typu 2; CBD: kannabidiol; CHTX: charybdotoksyna, inhibitor $K_{Ca}1.1$ i $K_{Ca}3.1$; CID16020046: antagonista receptora GPR55; COX-1: cyklooksygenaza-1; COX-2: cyklooksygenaza-2; EA: etanoloamina; eCB: śródbłonkowy receptor kannabinoidowy; eNOS: śródbłonkowa syntaza tlenku azotu; EP_4: receptor prostaglandynowy EP4; FAAH: hydrolaza amidu kwasów tłuszczowych; GPR18: sierocy receptor sprzężony z białkiem G18; GPR55: sierocy receptor sprzężony z białkiem G55; GW9662: antagonista receptora PPAR-γ; IBX: iberiotoksyna, inhibitor $K_{Ca}1.1$; INDO: indometacyna, inhibitor COX-1/COX-2; IP: receptor prostacyklinowy; JZL184: inhibitor lipazy monoacyloglicerolowej; K_{Ca}: kanały potasowe aktywowane wapniem; $K_{Ca}2.3$, $K_{Ca}3.1$ i $K_{Ca}1.1$: kanały potasowe aktywowane wapniem o małej, pośredniej i dużej przewodności dla K^+; L-NAME: inhibitor eNOS; LPI: L-alfa-lizofosfatydyloinozytol; L161982: antagonista receptora EP_4; MAGL: lipaza monoacyloglicerolowa; nimesulid: inhibitor COX-2; O-1918: antagonista receptora eCB; pEC_{50}: ujemny logarytm z molowego stężenia agonisty powodującego połowę efektu (skurczu/rozkurczu)

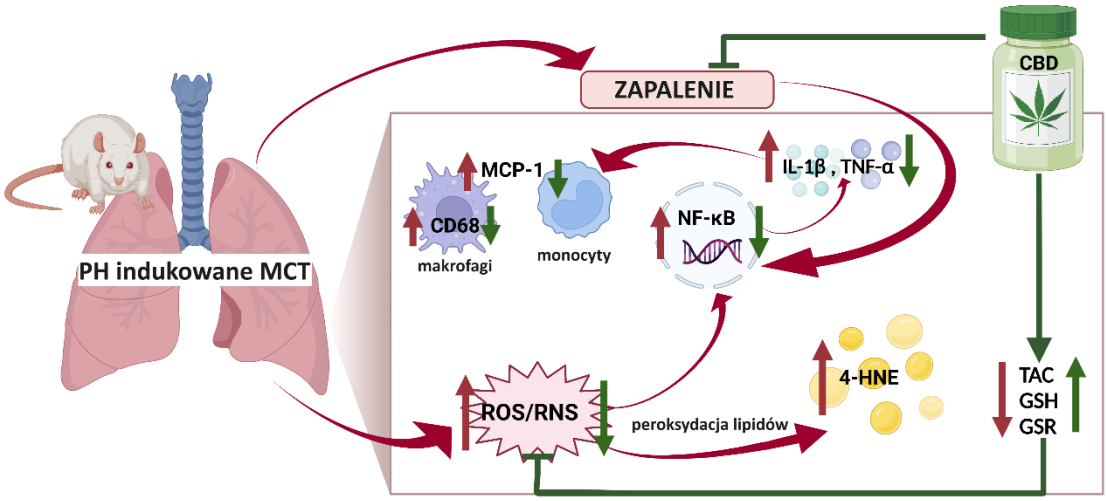
maksymalnego; PGE_2: prostaglandyna E2; PGI_2: prostacyklina; PPAR-γ: receptor aktywowany przez proliferatory peroksysomów gamma; RO1138452: antagonista receptora IP; SR141716: rimonabant, antagonista receptora CB_1; SR144528: antagonista receptora CB_2; TEA: inhibitor $K_{Ca}2.3$ i $K_{Ca}3.1$; TRAM-34: inhibitor $K_{Ca}2.3$; TRPV1: receptor waniloidowy przejściowego potencjału 1; TXA_2: tromboksan A_2; UCL1684: inhibitor $K_{Ca}2.3$; URB597: inhibitor FAAH; VIR: wirodamina. Rycinę przygotowano przy użyciu szablonu ze strony internetowej Servier Medical Art.

5.3.2. Kannabidiol wykazuje działanie przeciwzapalne i antyoksydacyjne w płucach szczurów z nadciśnieniem płucnym indukowanym monokrotaliną

Obiecujące wyniki badań nad kannabinoidami w łożysku płucnym opisane w rozdziale powyżej (w tym szczególnie wyniki z naszej pracowni nad CBD), a także doniesienia o potencjale przeciwzapalnym i przeciwutleniającym CBD przyczyniły się do postawienia tezy, że CBD może być związkiem o korzystnym działaniu plejotropowym w uzupełniającym leczeniu PAH. Zgodnie z doniesieniami, że terapia skojarzona zmniejsza ryzyko pogorszenia stanu klinicznego pacjentów, Europejskie Towarzystwo Kardiologiczne (ESC) i ERS zalecają polifarmakologię, czyli stosowanie leków (lub kombinacji leków), które mają wielokierunkowe działanie w terapii PAH (Galie i wsp. 2016). W związku z tym, celem mojej pierwszej pracy oryginalnej było zbadanie wpływu przewlekłego podawania CBD na parametry stresu oksydacyjnego i stanu zapalnego w płucach szczurów z PH indukowanym MCT.

W płucach szczurów z PH indukowanym MCT wykazałam: zmniejszenie poziomu parametrów antyoksydacyjnych tj.: TAC i GSH, wzrost ilości mediatorów zapalnych tj.: NF-κB, TNF-α, IL-1β, MCP-1 i CD68 oraz nasilenie immunoekspresji receptorów CB_1 i CB_2. Wymienione zmiany potwierdzają rozwinięte PH (Rycina 6). Podawanie CBD szczurom z PH indukowanym MCT, prowadziło do zwiększenia poziomów TAC i GSH, redukcji ilości receptorów CB_1, których aktywacja prowadzi do działania prozapalnego i prooksydacyjnego, a także zmniejszało poziomy mediatorów stanu zapalnego NF-κB, TNF-α, IL-1β, MCP-1 i CD68 (Rycina 6). Nasilony stres oksydacyjny i stan zapalny indukują przebudowę naczyń płucnych czego konsekwencją jest nadmierne zwężenie naczyń płucnych i wzrost płucnego oporu naczyniowego (Mandras i wsp. 2020; Evans i wsp. 2020). Zmniejszona aktywność antyoksydacyjna jest ściśle powiązana z patogenezą PAH, a poziomy ROS są trzykrotnie wyższe w osoczu pacjentów z PAH (Wong i wsp. 2013). Zapalenie okołonaczyniowe wiąże się ze wzrostem ilości mediatorów zapalnych w tym NF-κB, TNF-α, IL-1β i CD68 co koreluje z postępem choroby (Hu i wsp. 2020; Zawia i wsp. 2021). Moje wyniki badań potwierdzają, że przewlekłe podawanie CBD reguluje równowagę oksydoredukcyjną i stan zapalny, a ponadto sugerują, że właściwości te są powiązane ze szlakiem NF-κB. Szlak NF-κB uczestniczy w aktywacji genu *MCP-1*. W moich badaniach zmniejszenie ilości NF-κB po podawaniu CBD korespondowało ze spadkiem poziomu parametru MCP-1, a także mediatorów zapalenia TNF-α i IL-1β w tkance płucnej. W związku z powyższym można przypuszczać, że hamowanie szlaku NF-κB przez CBD wiąże się ze zmniejszoną

infiltracją monocytów i makrofagów do tkanki okołonaczyniowej płuc ograniczając rozwój stanu zapalnego, a co za tym idzie, niekorzystną przebudowę naczyń płucnych. Warto zaznaczyć, że CBD nie wpływa na parametry stresu oksydacyjnego i stanu zapalnego u zdrowych zwierząt (CTR). Wydaje się zatem, że działanie CBD może ograniczać się do stanów patologicznych, pozostając neutralnym u zdrowych zwierząt co potwierdza jego dobry profil bezpieczeństwa. Podsumowanie wyników badań znajduje się na Rycinie 6, a szczegółowy opis w pracy oryginalnej w Rozdziale 11.



Rycina 6. Proponowany mechanizm działania kannabidiolu w płucach szczurów z nadciśnieniem płucnym indukowanym monokrotaliną. Skróty: 4-HNE: 4-hydroksynonenal; CBD: kannabidiol; CD68: antygen różnicowania komórkowego 68; GSH: glutation; GSR: reduktaza glutationowa; IL-1β: interleukina 1 beta; MCP-1: białko chemotaktyczne monocytów 1; MCT: monokrotalina; NF-κB: jądrowy czynnik transkrypcyjny kappa B; PH: nadciśnienie płucne; RNS: reaktywne formy azotu; ROS: reaktywne formy tlenu; TAC: całkowita pojemność antyoksydacyjna; TNF-α: czynnik martwicy nowotworu alfa. Stworzone w Biorender.com.

5.3.3. Kannabidiol posiada potencjał przeciwzwłóknieniowy w prawej komorze serca szczurów z nadciśnieniem płucnym indukowanym monokrotaliną

Najpoważniejszą konsekwencją zmian w naczyniach płucnych w przebiegu PAH jest wzrost oporu w krążeniu płucnym, co przekłada się na nadmierne obciążenie następcze prawej komory serca i jej niewydolność, prowadzi do pogorszenia rokowania i przedwczesnej śmierci pacjentów (Hassoun 2021). Długotrwałe wysokie obciążenie następcze z towarzyszącym stanem zapalnym i stresem oksydacyjnym przyczyniają się do patologicznej przebudowy i zwłóknienia prawej komory (Andersen i wsp. 2019). Podczas tego procesu aktywowanych jest wiele szlaków sygnałowych, w tym szlak TGF-β1/SMAD2. Następuje także zwiększona aktywacja fibroblastów, które przekształcają się w miofibroblasty i wytwarzają elementy macierzy zewnątrzkomórkowej (Frangogiannis, 2017; Egemnazarov i wsp. 2018). Ponadto charakterystyczne jest zwiększenie ekspresji markerów dysfunkcji i/lub przebudowy serca, takich jak NT-proBNP (Benza i wsp. 2019) czy α-SMA i Gal-3 w prawej komorze (He i wsp. 2017; Egemnazarov i wsp. 2018). Rozległa przebudowa i zwłóknienie prawej komory wiążą się z utratą dużej liczby kardiomiocytów, ostatecznie zastępując martwy mięsień sercowy blizną kolagenową, co wpływa na pogorszenie jej funkcji skurczowo-rozkurczowej.

Biorąc pod uwagę, że niewydolność prawej komory w PAH jest jednym z czynników przyczyniających się do wysokiej śmiertelności, a dostępne leki nie pozwalają na całkowite wyleczenie PAH i ich głównym efektem jest rozszerzenie naczyń płucnych, celem mojej drugiej pracy oryginalnej było sprawdzenie, czy CBD posiada potencjał przeciwzwłóknieniowy w prawej komorze szczurów z PH indukowanym MCT.

W badaniach u szczurów z PH indukowanym MCT, wystąpiły zmiany w prawej komorze, potwierdzające jej rozwiniętą dysfunkcję m.in. zwiększoną/e: szerokość kardiomiocytów, powierzchnię zwłóknienia śródmiąższowego i okołonaczyniowego, poziomy fibroblastów i fibronektyny, a także zwiększony poziom parametrów TGF-β1, Gal-3, pSMAD2, SMAD2 i α-SMA. Poziom VE-kadheryny, która jest markerem prawidłowego śródbłonka, był niższy w prawych komorach serc szczurów z PH indukowanym MCT. W prawych komorach szczurów poddanych podwiązaniu tętnicy płucnej (co wiąże się ze zwiększonym obciążeniem następczym prawej komory) również zaobserwowano zwiększone poziomy TGF-β1, SMAD2 i pSMAD2 (Sun i wsp. 2018). Fakt ten i wyniki badań własnych mogą przemawiać na korzyść modelu PH indukowanego MCT, w którym podobny kierunek zmian tych parametrów prozwłóknieniowych jest prawdopodobnie spowodowany przeciążeniem prawej komory

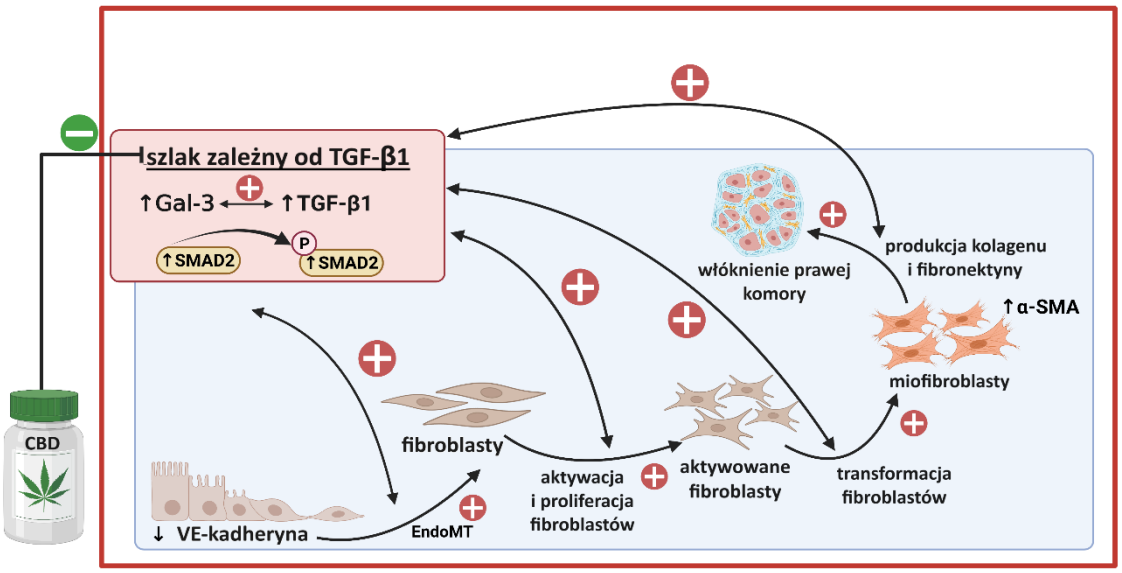
serca, podobnie jak w przypadku PAH u ludzi. Dodatkowo moje badania ujawniają szczególną rolę szlaku TGF-β1/SMAD2 w zwłóknieniu prawej komory w modelu PH indukowanym MCT u szczurów. Spadek poziomu VE-kadheryny przy jednoczesnym wzroście poziomu parametrów TGF-β1, SMAD2, pSMAD2 i α-SMA sugeruje udział szlaku TGF-β1/SMAD2 w procesie EndoMT i przebudowy prawej komory serca w PH indukowanym MCT. Oprócz powyższego Fenster i wsp. (2016) zasugerowali, że Gal-3 może być dobrym osoczowym markerem PAH, który odzwierciedla postęp choroby, a w badaniach własnych zaobserowano znacznie podwyższony poziom Gal-3 w prawych komorach serca szczurów z PH, co potwierdza te doniesienia. Ostatnie badania sugerują ścisły synergistyczny związek między Gal-3 a szlakiem TGF-β1/SMAD. Egzogennie podana Gal-3 wzmaga syntezę TGF-β1, SMAD2 i kolagenu I w fibroblastach przedsionków serca, a egzogennie dodany TGF-β1 wzmaga syntezę Gal-3 (Xiao i wsp. 2020). Wydaje się zatem, że Gal-3 bierze udział w procesie przebudowy prawej komory poprzez interakcję ze szlakiem TGF-β1/SMAD2.

W eksperymentach po raz pierwszy wykazałam, iż przewlekłe podawanie CBD obniża stężenie jednego z najczęściej stosowanych obecnie markerów niewydolności serca, tj. NT-proBNP w osoczu szczurów z PH indukowanym MCT. Wydaje się to mieć duże znaczenie kliniczne, gdyż obniżenie stężenia NT-proBNP w osoczu u chorych leczonych sildenafilem i bozentanem korelowało z lepszą odpowiedzią na leczenie pacjentów z PAH (Sharif-Kashani i wsp. 2014). Dodatkowo przewlekłe podawanie CBD zmniejszyło szerokość kardiomiocytów oraz obszar zwłóknienia śródmiąższowego i okołonaczyniowego, a także ilość markerów włóknienia, takich jak ilość fibroblastów i fibronektyny, poziomy TGF-β1, Gal-3, SMAD2 i pSMAD2 w prawych komorach u szczurów z PH indukowanym MCT.

Szlak sygnałowy TGF-β1/SMAD2 jest jednym z głównych aktywatorów zwłóknienia prawej komory w PH indukowanym MCT, a przewlekłe podawanie CBD zmniejszyło poziomy białek TGF-β1, SMAD2 i pSMAD2. Z powyższych badań wynika, że CBD prawdopodobnie hamuje szlak TGF-β1/SMAD2 w prawej komorze, a tym samym zapobiega jej przebudowie. Zahamowanie szlaku TGF-β1/SMAD spowodowało poprawę czynności serca i zmniejszenie jego przebudowy w szczurzym modelu zwłóknienia serca wywołanego dietą wysokosolną (Hu i wsp. 2023). Xiao i wsp. (2020) opisali powiązanie szlaku TGF-β1/SMAD2 z Gal-3. W badaniach własnych kierunek zmian tych parametrów w prawej komorze po podaniu CBD jest podobny, co potwierdza, że docelowym punktem działania CBD może być szlak TGF-β1/SMAD2.

Dodatkowo hamowanie Gal-3 przez CBD w prawej komorze może mieć działanie protekcyjne w PH, ponieważ zaobserwowano mniejszy wzrost RVSP i zmniejszoną przebudowę prawej komory serca u myszy Gal-3$^{-/-}$ eksponowanych na niedotlenienie w porównaniu z myszami typu dzikiego z niedotlenieniem (Hao i wsp. 2017).

Na rycinie 7 podsumowano wyniki badań w prawych komorach serca szczurów z PH indukowanym MCT, a także potencjalne punkty uchwytu działania CBD.



Rycina 7. Podsumowanie procesu zwłóknienia prawej komory serca w nadciśnieniu płucnym indukowanym monokrotaliną. W patologicznym procesie przebudowy serca aktywowane fibroblasty przekształcają się w miofibroblasty, które wytwarzają macierz zewnątrzkomórkową (kolagen, fibronektyna). Miofibroblasty indukują lokalną aktywację transformującego czynnika wzrostu β1 (TGF-β1), który reguluje odkładanie się macierzy zewnątrzkomórkowych poprzez, m.in. aktywację szlaku kanonicznego SMAD. TGF-β1 stymuluje aktywację i transformację fibroblastów w miofibroblasty, co wyraża się zwiększoną ekspresją alfa-aktyny mięśni gładkich (α-SMA), jednak taki efekt jest możliwy w wyniku wcześniejszej fosforylacji SMAD2 (Goumans i wsp. 2018). Przejście endotelialno-mezenchymalne (EndoMT) to proces, w którym komórki śródbłonka przekształcają się w komórki mezenchymalne lub mięśnie gładkie, a szlak TGF-β1/SMAD odgrywa w tym procesie kluczową rolę (Egemnazarov i wsp. 2018). TGF-β1 oprócz aktywacji komórek śródbłonka do wytwarzania mediatorów zwłóknienia, indukuje także EndoMT. Podczas progresji EndoMT komórki śródbłonka tracą markery specyficzne dla śródbłonka, takie jak VE-kadheryna, i nabywają markery mezenchymalne podobne do fibroblastów, w tym α-SMA (Yao i wsp. 2018). Architektura tkanek zostaje naruszona i następuje odkładanie tkanki włóknistej. Konsekwencją tych procesów jest nadmierne zesztywnienie prawej komory oraz pogorszenie jej funkcji skurczowo-rozkurczowej (Yao i wsp. 2020). Stworzone w Biorender.com.

Podsumowując badania własne i przegląd literatury, CBD wykazuje plejotropowe, korzystne działanie w tym: rozszerzające naczynia płucne, działanie antyoksydacyjne i przeciwzapalne w tkance płucnej i działanie przeciwzwłóknieniowe w prawej komorze szczurów z PH indukowanym MCT. Tym samym CBD może znaleźć w przyszłości zastosowanie jako terapia uzupełniająca w leczeniu PAH, wymaga to jednak potwierdzenia w dalszych badaniach eksperymentalnych i klinicznych, ze szczególnym uwzględnieniem i ukierunkowaniem na opisane przeze mnie i inne szlaki sygnałowe zaangażowane w patogenezę PAH.

Rozdział 6. Wnioski

1. (Endo)kannabinoidy, w tym CBD, odgrywają rolę w regulacji napięcia naczyń płucnych poprzez mechanizmy zależne i niezależne od śródbłonka, w tym mechanizmy receptorowe (CB_1, eCB, IP, EP_4, GPR18, PPAR-γ, TRPV1) i/lub mechanizmy pozareceptorowe m.in. szlaki enzymatyczne (FAAH, MAGL, COX-1, COX-2), co może przyczyniać się do zmniejszenia oporu płucnego i odgrywać ważną rolę w terapii wspomagającej leczenie PAH.

2. Kannabidiol w modelu eksperymentalnym PH indukowanym MCT u szczurów wykazuje działanie:

- antyoksydacyjne i przeciwzapalne w tkance płucnej za pośrednictwem hamowania szlaku NF-κB i zmniejszenia poziomu receptorów CB_1, których aktywacja nasila działanie prooksydacyjne i prozapalne,
- przeciwzwłóknieniowe w prawej komorze serca za pośrednictwem osłabiania szlaku sygnalizacyjnego TGF-β1/SMAD2.

3. Wielokierunkowe korzystne działanie CBD w modelu doświadczalnym PH indukowanym MCT wpisuje się w obecny trend terapii celowanej w kilka punktów uchwytu leczenia PAH, w tym rozkurcz naczyń płucnych, zmniejszanie stanu zapalnego, stresu oksydacyjnego i działanie przeciwzwłóknieniowe. Obiecujące wyniki doświadczalne wskazują na potrzebę szczegółowych badań eksperymentalnych i klinicznych nad wpływem CBD lub jego pochodnych w uzupełniającej terapii PAH.

Rozdział 7. Piśmiennictwo

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Rozdział 8. Streszczenie w języku polskim

Nadciśnienie płucne zdefiniowane jako wzrost średniego ciśnienia w tętnicy płucnej powyżej 20 mmHg, jest nieuleczalną chorobą o wieloczynnikowej etiologii. Obejmuje m.in. dysfunkcję śródbłonka naczyń płucnych prowadząc do ich nadmiernego skurczu i przebudowy, rozwój stanu zapalnego i stresu oksydacyjnego. W następstwie dochodzi do zwiększenia oporu w krążeniu płucnym, wzrostu obciążenia następczego, przerostu i zmian zwłóknieniowych prawej komory serca, jej niewydolności i przedwczesnej śmierci pacjenta. Obecna terapia nie pozwala na pełne wyleczenie choroby i wykorzystuje głównie leki o działaniu naczyniorozszerzającym. Sugeruje się, że najlepszą strategią terapeutyczną w leczeniu nadciśnienia płucnego jest wczesna politerapia celująca w kilka punktów uchwytu w tym: zmniejszenie oporu naczyniowego w krążeniu płucnym, przeciwdziałanie zmianom zwłóknieniowym i proliferacyjnym oraz działanie przeciwzapalne i antyoksydacyjne.

Kannabidiol (CBD) to jeden z najlepiej przebadanych składników *Cannabis sativa*, który nie wykazuje działania psychoaktywnego i odznacza się wysokim profilem bezpieczeństwa. Kannabidiol posiada szeroki zakres korzystnych właściwości obejmujących działanie przeciwzapalne, antyoksydacyjne, przeciwzwłóknieniowe i rozszerzające naczynia płucne.

W związku z powyższym, **celem badań** była ocena wpływu CBD na parametry zapalne i włóknienia w tkance płucnej i/lub prawej komorze serca w szczurzym modelu nadciśnienia płucnego (ang. *pulmonary hypertension* – PH) indukowanego alkaloidem roślinnym monokrotaliną (MCT) z jednoczesną analizą przydatności kannabinoidów w terapii tej jednostki chorobowej.

Doświadczenia zostały przeprowadzone na tkankach pochodzących od szczurów z PH indukowanym MCT (jednorazowa, podskórna iniekcja, 60 mg/kg). Kannabidiol (10 mg/kg) lub rozpuszczalnik podawano dootrzewnowo przez 21 dni od momentu iniekcji MCT. W pracy wykorzystano metody biochemiczne (m.in. Western Blot, ELISA) i histologiczne (m.in. barwienia immunohistochemiczne).

W płucach szczurów z PH indukowanym MCT stwierdzono spadek całkowitej pojemności antyoksydacyjnej (TAC) i poziomu glutationu (GSH), wzrost ilości mediatorów zapalnych tj.: czynnika martwicy nowotworu alfa (TNF-α), interleukiny 1 beta (IL-1β), jądrowego czynnika transkrypcyjnego kappa B (NF-κB), białka chemotaktycznego monocytów-1 (MCP-1) i antygenu różnicowania komórkowego 68

(CD68) oraz receptorów kannabinoidowych CB_1 i CB_2. W prawej komorze serc szczurów stwierdzono wzrost poziomu parametrów związanych z jej dysfunkcją oraz parametrów profibrotycznych w tym: peptydu natriuretycznego typu pro-B (NT-proBNP), szerokości kardiomiocytów, obszaru zwłóknienia śródmiąższowego i okołonaczyniowego, ilości fibroblastów i fibronektyny, jak również poziomu transformującego czynnika wzrostu beta 1 (TGF-β1), galektyny-3 (Gal-3), SMAD2, pSMAD2 i alfa-aktyny mięśni gładkich (α-SMA). Przeciwnie, poziom VE-kadheryny (marker komórek śródbłonka) był obniżony. Chroniczne podawanie CBD zwiększyło poziomy TAC i GSH, oraz zmniejszyło poziom receptorów CB_1 i czynników TNF-α, IL-1β, NF-κB, MCP-1 i CD68. Kannabidiol zredukował także stężenie NT-proBNP w osoczu, szerokość kardiomiocytów, wielkość obszaru zwłóknienia, poziomy fibronektyny i fibroblastów, a także poziom białek TGF-β1, Gal-3, SMAD2, pSMAD2 i zwiększył poziom VE-kadheryny w prawej komorze serc szczurów.

Podsumowując badania własne, CBD wykazuje plejotropowe, korzystne działanie w tym: antyoksydacyjne i przeciwzapalne w tkance płucnej oraz przeciwzwłóknieniowe w prawej komorze serc szczurów z PH indukowanym MCT. W połączeniu z działaniem rozszerzającym naczynia płucne, co zostało podsumowane w pracy przeglądowej, CBD wpisuje się w aktualne trendy leczenia nadciśnienia płucnego jako terapia uzupełniająca.

Rozdział 9. Streszczenie w języku angielskim/Summary

Pulmonary hypertension, defined as when mean pulmonary artery pressure is above 20 mmHg, is an incurable disease with a multifactorial etiology. The pathogenesis of PH involves endothelial dysfunction of pulmonary vessels leading to their excessive contraction and remodeling, increased inflammation and oxidative stress. The result of above-mentioned changes is increased resistance in the pulmonary circulation, increased afterload, hypertrophy and fibrotic changes of the right ventricle, its failure and premature patients' death. Current therapies do not fully cure the disease and cover mostly pulmonary vasorelaxation. It is suggested that the best therapeutic strategy for treating pulmonary hypertension is early polytherapy targeting several points of resolution including reducing vascular resistance in the pulmonary circulation, as well as anti-inflammatory, antioxidant, and anti-fibrotic effects.

Cannabidiol (CBD) is one of the best researched constituents of *Cannabis sativa*, with no psychoactive effects and a high safety profile. Cannabidiol has wide range of beneficial properties including anti-inflammatory, antioxidant, anti-fibrotic and vasorelaxant effect on pulmonary vessels.

Therefore, **the aim of the study** was to evaluate the effect of CBD on inflammatory and fibrotic parameters in lung tissue and/or right ventricle in a rat model of pulmonary hypertension (PH) induced by the plant alkaloid monocrotaline (MCT) with a simultaneous analysis of the usefulness of cannabinoids in adjuvant therapy for PH treatment.

The experiments were performed on tissues collected from rats with MCT-induced PH (single, subcutaneous injection, 60 mg/kg). Cannabidiol (10 mg/kg) or its solvent was administered intraperitoneally for 21 days after MCT injection. In the studies I used biochemical (including Western Blot, ELISA) and histological (including immunohistochemical staining) methods.

The lungs of rats with MCT-induced PH showed a decrease in total antioxidant capacity (TAC) and glutathione (GSH) levels, an increase in inflammatory mediators: tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), nuclear factor kappa B (NF-κB), monocyte chemoattractant protein-1 (MCP-1) and cluster of differentiation 68 (CD68), as well as cannabinoid receptors CB_1 and CB_2. The rats' right ventricles showed an increase in the levels of parameters related to its dysfunction and profibrotic parameters including: N-terminal pro b-type natriuretic peptide (NT-proBNP),

cardiomyocyte width, area of interstitial and perivascular fibrosis, number of fibroblasts and immunoexpression of fibronectin, as well as levels of transforming growth factor beta 1 (TGF-β1), galectin-3 (Gal-3), SMAD2, pSMAD2 and alpha-smooth muscle actin (α-SMA). On the contrary, VE-cadherin (a marker of endothelial cells) level was reduced. Chronic CBD administration increased the levels of TAC and GSH and decreased the levels of CB_1 receptors and the factors: TNF-α, IL-1β, NF-κB, MCP-1 and CD68. Cannabidiol also reduced plasma NT-proBNP concentrations, cardiomyocyte width, area of fibrosis, immunoexpression of fibronectin and fibroblast amount, as well as the levels of TGF-β1, Gal-3, SMAD2, pSMAD2 proteins and increased VE-cadherin levels in the right ventricles of rat.

In summary, my own research shows pleiotropic beneficial effects of CBD including antioxidant and anti-inflammatory in lung tissue and anti-fibrotic in the right ventricle of rats with MCT-induced PH. Above mentioned effects combined with pulmonary vasorelaxant effects, as summarized in the in the review paper, suggest that CBD fits in with current trends as an adjuvant therapy for pulmonary hypertension treatment.

Rozdział 10. Praca przeglądowa

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Review

Cannabinoids—A New Perspective in Adjuvant Therapy for Pulmonary Hypertension

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Abstract: Currently, no treatment can completely cure pulmonary hypertension (PH), which can lead to right ventricular failure and, consequently, death. Therefore, searching for new therapies remains important. Increased resistance in pulmonary circulation is mainly caused by the excessive contraction and proliferation of small pulmonary arteries. Cannabinoids, a group of lipophilic compounds that all interact with cannabinoid receptors, exert a pulmonary vasodilatory effect through several different mechanisms, including mechanisms that depend on vascular endothelium and/or receptor-based mechanisms, and may also have anti-proliferative and anti-inflammatory properties. The vasodilatory effect is important in regulating pulmonary resistance, which can improve patients' quality of life. Moreover, experimental studies on the effects of cannabidiol (plant-derived, non-psychoactive cannabinoid) in animal PH models have shown that cannabidiol reduces right ventricular systolic pressure and excessive remodelling and decreases pulmonary vascular hypertrophy and pulmonary vascular resistance. Due to the potentially beneficial effects of cannabinoids on pulmonary circulation and PH, in this work, we review whether cannabinoids can be used as an adjunctive therapy for PH. However, clinical trials are still needed to recommend the use of cannabinoids in the treatment of PH.

Keywords: pulmonary hypertension; cannabinoids; pulmonary vessels; vasorelaxation; vasoconstriction

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1. Introduction

Pulmonary hypertension (PH) refers to a group of clinical symptoms caused by increased blood pressure (BP) in the pulmonary circulation. According to the latest classification, PH is diagnosed, when the mean pulmonary artery pressure (mPAP) at rest is over 25 mmHg, confirmed by right-sided heart catheterization. The World Health Organization (WHO) distinguishes five groups of PH: pulmonary arterial hypertension (PAH), PH due to left-sided heart disease, PH due to chronic lung disease, chronic thromboembolic PH, and PH with unexplained and/or multifactorial pathomechanisms [1,2]. PH often leads to heart failure due to the excessive overload of the right ventricle (RV), which can result in the patient's death [3]. The development of PH is complex, and its pathogenesis can include the dysfunction of vascular endothelial cells with the excessive contraction of the pulmonary arteries, vascular, and RV remodelling (the proliferation of muscle cells and hypertrophy), inflammation, oxidative stress, and thrombosis [4–6].

The current treatments for PH include phosphodiesterase type 5 (PDE-5) inhibitors (e.g., sildenafil), soluble guanylate cyclase (sGC) stimulators (riociguat), endothelin receptor antagonists (ERAs) (e.g., bosentan), prostacyclin (PGI_2) analogues (e.g., iloprost), and prostacyclin receptor (IP) agonists (selexipag) [7]. Combination therapy has emerged as the contemporary standard of care in the treatment of PH patients who are mostly symptomatic. However, this therapy does not ensure the long-term normalization of

pulmonary resistance, which is an unfavorable prognostic factor. Researchers are currently seeking drugs that not only lower pulmonary resistance, but also have anti-proliferative properties [8]. There is currently no therapy that allows patients to fully recover, and PH is still characterized by high mortality [3]. Therefore, new compounds that act on signalling pathways with documented roles in the pathomechanisms of the disease are currently being sought. The first reports on the relaxing effects of cannabinoids on isolated human pulmonary vessels raised the following question: can cannabinoids be used in the treatment of PH? [9]. Hornig [10] hypothesized that cannabinoids could become an element of PH therapy but noted that we still have too little knowledge on this subject and that further experiments are needed. In this review, based on the latest reports, we explored this hypothesis in more detail.

Cannabinoids have been exploited for centuries for recreational and medicinal purposes. When smoked, cannabinoids mainly cause changes in the central nervous system. Moreover, reports suggest that cannabinoids influence the respiratory and circulatory systems. According to the United States Code (USC), marijuana is defined as all parts of the plant *Cannabis sativa* L. var. *indica* and contains about 700 compounds, more than 100 of which are cannabinoids, such as the psychoactive delta-9-tetrahydrocannabinol (THC), non-psychoactive cannabidiol (CBD), tetrahydrocannabivarin, and cannabidivarin. It is believed that marijuana has analgesic, anticonvulsant, and anti-asthmatic properties [11]. Research on the effects of plant-derived cannabinoids (phytocannabinoids) and mammalian-organism-produced endocannabinoids (arachidonic-acid derivatives) has recently received widespread interest. It is already known that the endocannabinoid system (ECS) is upregulated in some types of hypertension, including PH [12], and that the ECS components may have anti-proliferative effects [13].

The aim of this review was to determine what vascular mechanisms are involved in cannabinoid-induced pulmonary vasodilation and what effects of cannabinoids have been observed to date during in vivo studies (including experimental PH) to produce a preliminary evaluation of the usefulness of cannabinoids in the assisted treatment of PH. Another objective of this review was to examine the evidence from experimental and human studies showing what endothelium-dependent mechanisms and/or receptors are involved in cannabinoid-mediated responses in the pulmonary vasculature, including cannabinoid receptors types 1 and 2 (CB_1-Rs and CB_2-Rs), historically called endothelial cannabinoid receptors (eCB-Rs), transient receptor potential vanilloids 1 and 4 (TRPV1 and TRPV4), peroxisome proliferator-activated receptors-γ (PPAR-γ), and prostanoid receptors. This review only briefly describes the effects of cannabinoids on systemic vessels, as these effects have been discussed in detail in reviews by Stanley et al. [14] and Bondarenko [15].

2. Cannabinoids in the Cardiopulmonary System

Cannabinoids are a group of lipophilic compounds that all interact with cannabinoid receptors (CB-Rs). The current classification of cannabinoids is based on their origin: phytocannabinoids isolated, for example, from *Cannabis sativa* L. var. *indica* (e.g., THC and CBD); compounds obtained via chemical synthesis (e.g., abnormal cannabidiol (Abn-CBD); WIN 55,212-2); and components of the ECS, such as endocannabinoids (e.g., 2-arachidonoylglycerol (2-AG), N-arachidonoylethanolamine (anandamide; AEA), and virodhamine (VIR)) and endocannabinoid-like molecules (e.g., noladin ether (2-AGE), N-arachidonoyl-L-serine (ARA-S), oleamide (ODA), and L-alpha-lysophosphatidylinositol (LPI)) [16,17]. The presence of all the components of the ECS in the lungs and pulmonary vessels of animals and humans was previously confirmed by various methods (see Table 1).

Table 1. Expression of the selected components of the endocannabinoid system in pulmonary circulation/lung tissue.

Endocannabinoid System Components		Material	Species	Methods	Expression			References
					Endothelium	Whole Vascular Wall	Whole Lung	
ligands	2-AG	lung cellular extracts	rabbit	LC/MS			+	[18]
		lung	rat				+	[19]
	AEA	lung cellular extracts	rabbit	LC/MS			+	[18]
		lung	rat	LC/MS			+	[19]
			mouse	LC/MRM			+	[20]
receptors	CB_1-R	pulmonary arteries	rat	IHC		+		[21]
				WB		+		
			human	WB		+		
				IHC		+		
						+		[22]
	CB_2-R	pulmonary arteries	human	IHC		+		[22]
				WB		+		[21]
			rat			+		
	TRPV1	pulmonary arteries	human	IHC		+		[22]
	GPR18	pulmonary arteries	human	IHC		+		[22]
	GPR55	pulmonary arteries	human	WB		+		[23]
				IHC	+			
enzymes	FAAH	pulmonary arteries	human	WB		+		[24]
		lung	human	WB			+	
			mouse				+	[20]
			rabbit	RT-PCR			+	[18]

+ expression detected. Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; CB_1-R, cannabinoid receptor type 1; CB_2-R, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; GPR18, G-protein-coupled receptor 18; GPR55, G-protein-coupled receptor 55; IHC, immunohistochemistry; LC/MS, liquid chromatography-mass spectrometry; LC/MRM, liquid chromatography-multiple reaction monitoring; RT-PCR, real-time polymerase chain reaction; TRPV1, transient receptor potential vanilloid 1; WB, western blot.

The ECS components include, for example, the classic G-protein-coupled cannabinoid receptors CB_1-R and CB_2-R. The presence of CB_1-Rs in the brain, liver, reproductive system, skeletal muscles, and cardiovascular system, including pulmonary vessels, has been confirmed [12,21,22,25]. CB_2-Rs have been found in the brain, spleen, and mainly immune system cells [12,25–28]. Cannabinoids also exert their effects through other receptors such as TRPV1, TRPV4, and PPAR-γ, as well as the G-protein-coupled orphan receptors GPR18, GPR55, and eCB-Rs which are O-1918-sensitive and have not yet been cloned [29]. Endocannabinoids are mainly produced "on demand" through the synthesis of membrane phospholipid precursors [25,30]. Enzymes from the group of diacylglycerol lipases (DAGLs)—DAGL-α and DAGL-β—participate in the synthesis of AEA and 2-AG, respectively. 2-AG is degraded in the pulmonary circulation mainly by the enzyme monoacylglycerol lipase (MAGL), and AEA is mainly degraded by fatty acid amide hydrolase (FAAH) into arachidonic acid (AA) (Table 1) [30–32].

Cannabinoids directly exert multidirectional effects on the vascular bed, including pulmonary vessels, through interactions with appropriate receptors and indirectly through the metabolites resulting from the degradation of (endo)cannabinoids. The degradation of endocannabinoids primarily produces AA, which is converted into eicosanoids via the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (cytP450) pathways. The COX pathway that mediates the formation of PGI_2, prostaglandins (PG), and thromboxane A2 (TXA_2) plays the most important role in vascular responses [33]. Moreover, Sadowska et al. [19] recently demonstrated the presence of 13 endocannabinoids and endocannabinoid-related lipids in the lungs of control and monoctrotaline (MCT)-induced PH rats. These 13 endocannabinoids were AEA, 2-AG, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), stearoyl ethanolamide (SEA), inolenoyl ethanolamide (LEA), palmitoleoyl ethanolamide (POEA), N-arachidonoylglycine (NAGly), docosahexaenoyl ethanolamide (DHEA), docosatetraenoyl ethanolamide (DEA), homo-γ-linolenyl ethanolamide (HEA), linoleoylglycerol (2-LG), and eicosapentaenoyl ethanolamide (EPEA), among which OEA, SEA, HEA, DEA, 2-LG, DHEA, POEA, and EPEA were examined for the first time. To date, however, little research has explored the role of the above-mentioned endocannabinoids in the physiology and pathophysiology of the pulmonary circulation.

3. Effects of Cannabinoids on Systemic Vessels

The ECS is unlikely to be the main element regulating the cardiovascular parameters in physiological conditions, although it plays an important role in pathological states [29,34]. The effects of cannabinoids on blood vessels have been studied since the 1990s, and new research continues to emerge. Cannabinoids in systemic circulation cause the relaxation of the blood vessels, which was extensively described by Stanley et al., in 2014. In this review, we focus on papers published after Stanley et al. (Table 2) [14]. The relaxation induced by various cannabinoids might be dependent on the endothelium [22,35–42] and/or receptors (e.g., CB_1-Rs) [22,35,37,38,40–43] (Table 2). The potency of individual compounds depends on the vascular bed and species. As shown in Table 2, according to the negative logarithm of the concentration causing a half-maximum effect (pEC_{50}) value, methanandamide (MethAEA) [42] dilated rat mesenteric arteries (rMAs) most strongly, while the weakest effects were observed for Abn-CBD in rat retinal capillaries [44] and arachidonyl cyclopropylamide (APCA) in rat aortas [43].

Table 2. The relaxing effects of cannabinoids on systemic vessels (published after 2014).

Ligand	Blood Vessel	pEC_{50}	Mechanisms								References
			Endo	eNOS	COX	K_{Ca}	CB_1-R	CB_2-R	eCB	Other	
AEA	hMA	5.7	↓	↓	**No**	-	↓	**No**	↓		[38]
	rRet	5.2	-	-	-	-	-	-	-		[40]
2-AG	rRet	5.0	-	-	-	-	-	-	-		[40]
	rMA	5.9 *	↓	-	**No**	↓	**No**	**No**	-	TRPV4	[36]
2-AGE	rMA	5.6 *	**No**	-	-	-	-	-	-		[36]
NAGLy	rMA	-	↓	↓	**No**	**No**	**No**	**No**	↓		[39]
CBD	hMA	5.1	↓	↓	**No**	↓	↓	**No**	**No**	TRPV1	[37]
	rFA [1]	-	**No**	↓	↓	-	**No**	↓	**No**	SOD, EP_4	[45]
	rFA, rA [1]	-	-	↓	↓	-	-	-	-		[46]
	rMA [1]	-	-	**No**	**No**	-	-	-	-		[46]
	rMA [2]	6.0	**No**	-	-	-	**No**	**No**	-		[22]
	rMA [3]	5.5	**No**	-	-	-	**No**	**No**	-		[22]
	rMA [4]	5.9	↓	-	-	-	↓	↓	-		[22]
	rMA [5]	5.6	**No**	-	-	-	↓	**No**	-		[22]
Abn-CBD	rRet	4.5	↓	**No**	-	↓	**No**	**No**	-		[44]
	pRet	-	↓	-	-	-	↓	-	↓		[35]
WIN 55,212-2	rRet	5.0	↓	↓	**No**	-	↓	**No**	**No**		[40]
JHW-133	rMA	-	-	↓	-	-	-	↓	-		[41]
MethAEA	rA [6]	6.1	-	-	-	-	-	-	-		[47]
	rMA [6]	4.9	-	-	-	-	**No**	-	-	TRPV1	[47]
	rMA [4]	5.6	-	-	-	-	↓	-	-	TRPV1	[47]
	rMA [5]	5.6	-	-	-	-	↓	-	-		[42]
	rMA [2]	6.1	-	-	-	-	**No**	-	-		

Table 2. *Cont.*

Ligand	Blood Vessel	pEC_{50}	Mechanisms								References
			Endo	eNOS	COX	K_{Ca}	CB_1-R	CB_2-R	eCB	Other	
ACPA	rA	4.3	**No**	-	-	↓	**No**	-	-	Ca_v 1.2	[43]
	rMA	-	↓	↓	-	↓	↓	-	-		[41]

[1] Zucker diabetic fatty rats; [2] WKY, Wistar-Kyoto rats; [3] SHAM, control sham-operated rats; [4] rats with secondary hypertension induced by Deoxycorticosterone acetate-salt (DOCA salt); [5] SHR, spontaneously hypertensive rats; [6] UNX, uninephrectomized normotensive rats; * pEC_{40}; ↓, weakening effect; **No**, no effect; **-**, not determined. Abbreviations: 2-AG, 2-arachidonoylglycerol; Abn-CBD, abnormal cannabidiol; ACPA, arachidonylcyclopropylamide; AEA, anandamide; ARA-S, N-arachidonoyl L-serine; Ca_v 1.2, voltage-dependent L-type calcium channel subunit alpha-1C; CB_1-R, cannabinoid receptor type 1; CB_2-R, cannabinoid receptor type 2; CBD, cannabidiol; COX, cyclooxygenase; eCB, historically called endothelial cannabinoid receptor; endo, endothelium; eNOS, endothelial nitric oxide synthase; EP_4, prostanoid EP_4 receptor; FAAH, fatty acid amide hydrolase; hMA, human mesenteric artery; JHW-133, 3-(1,1-dimethylbutyl)-6aR,7,10,10aR-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran, synthetic cannabinoid; K_{Ca}, calcium-activated potassium channels; MethAEA, methanandamide; NAGLy, N-Arachidonylglycine; pEC_{50}, the negative logarithm of the concentration causing a half-maximum effect; pRet, pig retinal arterioles, PTX: pertussis toxin; rA, rat aorta; rCA, rat coronary artery; rFA, rat femoral artery; rMA, rat mesenteric artery; rRet, rat retinal capillaries; SOD, superoxide dismutase; TRPV1, transient receptor potential vanilloid 1; TRPV4, transient receptor potential vanilloid 4; WIN 55,212-2, [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate, synthetic cannabinoid; VIR, virodhamine.

4. The Systemic Versus Pulmonary Circulation

As mentioned above, the ECS is located in the pulmonary circulation (for reviews, see Kicman and Toczek [29] and Karpińska et al. [48]) (Table 1), and its endocannabinoid components can cause the relaxation of systemic vessels, resulting in a decrease in BP [12]. Therefore, the question emerges as to whether these components could also have hypotensive effects in the pulmonary circulation. Moreover, cannabinoids can be administered by inhalation. From a pharmacological point of view, this method of delivery could accelerate the effects of their action in the pulmonary circulation. In considering this question, attention should be paid to the similarities and differences between systemic vessels and pulmonary vessels, as such factors can affect the mechanisms of action of cannabinoids. An extremely important element in the structure of pulmonary vessels is the endothelium, which, despite being a mechanical barrier also participates in maintaining proper vascular tone through the synthesis of vasoactive compounds [49]. In PH, there is a notable change in the endothelial synthesis of compounds regulating vascular tone, with a predominance of vasoconstrictors (TXA_2, angiotensin II (ANG II), 5-hydroxytryptamine (5-HT), and endothelin 1 (ET-1)) compared to vasodilators (nitric oxide (NO) and PGI_2) [4]. Under normal conditions, the pulmonary circulation is a low-pressure, low-resistance, and high-volume system. One of the most important features distinguishing the systemic circulation from pulmonary circulation is the presence of a mechanism that dilates blood vessels in response to hypoxia. Systemic arteries relax with decreased oxygen concentration, while pulmonary vessels constrict in response to hypoxia and increased blood oxygenation, transporting blood to more heavily oxygenated areas. Hypoxia induces hypoxic pulmonary vasoconstriction (HPV) and a hypoxic ventilatory response [50]. If hypoxia is prolonged, as can be the case in various chronic lung diseases, the spasm is accompanied by a remodelling of the vascular system leading to an increase in pulmonary vascular resistance (PVR) and the development of PH. In addition to hypoxia, the susceptibility to develop PH can also be increased by other genetic and environmental factors, even in the absence of a hypoxic stimulus [51].

5. Cannabinoids Affect Pulmonary Circulation

Similarly, as in the systemic circulation, cannabinoids are also shown to have a vasodilating effect in isolated pulmonary vessels (Table 3) [9,21–24,52–54]. Cannabinoids show a concentration-dependent vasodilating effect in human pulmonary arteries (hPAs). As shown in Table 3, according to the pEC_{50} value in hPAs, LPI [23] has the strongest vasodilatory effect (6.4), while 2-AG [21], AEA (in the presence of AM251) [9], and VIR [53] have similar levels of potency (approximately 5) and Abn-CBD [9] is the least potent. Similar results were obtained in animal pulmonary vessels (see Table 3).

Table 3. The vasorelaxant effects of cannabinoids on the pulmonary vessels.

Species	Ligands	Vasoconstrictor	pEC_{50}	Concentration [µmol/L]	Endothelium	Inhibitors					K_{Ca} Inhibitors					Antagonists								References
						eNOS	FAAH	COX-1 COX-2	COX-2	MAGL	KCl [60/120 mM]	$K_{Ca}1.1$ $K_{Ca}3.1$	$K_{Ca}1.1$	$K_{Ca}2.3$	$K_{Ca}3.1$	CB_1-R	CB_2-R	eCB	IP	EP_4	TRPV1	PPAR-γ	
						L-NAME	URB597	INDO	NIMES	JZL184		CHTX	IBTX	UCL164/ APA *	TRAM-34	AM251/ SR141716 *	AM630/ SR144528 *	O-1918	RO1138452	L161982	CAPS	GW9662	
human	**AEA** [1]	5-HT	5.2	0.1–100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[9]
	AEA	U46619	5.0	0.1–100	↓	↓	↓	↓	↓	-	↓	-	↓	-	-	No	No *	↓	↓	-	No	-	[24]
	VIR	5-HT	5.1	0.1–100	↓	↓ [3]	↓	↓	-	-	↓	↓	-	↓ *	-	No	No *	↓ 6.3 [2]	-	-	No	-	[53]
	2-AG	U46619	5.4	0.01–30	↓	-	No	-	-	↑	-	-	-	-	-	↓ 6.9 [2]	-	-	-	-	-	-	[21]
	LPI	Phe	6.4	0.01–3	↓	↓	-	No	-		↓	-	↓	↓	↓	No	-	↓ 5.8 [2]	-	-	-	↓	[23]
	CBD	U46619	5.0	0.1–30	↓	No	-	↓	↓	-	↓	-	↓	↓	↓	No	No	No	↓ 5.8 [2]	↓ 6.6 [2]	↓	↓	[22]
	Abn-CBD	5-HT	4.8	0.1–100	↓	No	-	No	-	-	↓	↓	-	↓ *	-	-	-	↓ 5.1 [2]	-	-	-	-	[9]
rabbit	**2-AGE**	pCa 6.3	-	0.1–3	↓	-	-	-	-	-	-	-	-	-	-	↓/ ↓ *	-	↓	-	-	-	-	[52]
	Abn-CBD	pCa 6.3	-	0.01–0.3	↓	-	-	-	-	-	-	-	-	-	-	↓/ ↓ *	-	↓	-	-	-	-	[52]

Table 3. *Cont.*

Species	Ligands	Vasoconstrictor		Concentration [μmol/L]	Endothelium		Inhibitors				K_{Ca} Inhibitors					Antagonists					References
			pEC_{50}		KCl [60/120 mM]		L-NAME	URB597	INDO	NIMES	JZL184	CHTX	IBTX	UCL1684/ APA *	TRAM-34	AM251/ SR141716 *	AM630/ SR144528 *	O-1918	RO1138452	L161982	CAPS	GW9662	
							eNOS	FAAH	COX-1 COX-2	COX-2	MAGL	$K_{Ca}1.1$ $K_{Ca}3.1$	$K_{Ca}1.1$	$K_{Ca}2.3$	$K_{Ca}3.1$	CB_1-R	CB_2-R	eCB	IP	EP_4	TRPV1	PPAR-γ	
rat	**AEA**	U46619	5.0	0.1–100	↑	↑	↑	↑	↑	-	-	↑	-	↑*	-	**No**	**No**	↓ 6.0 [2]	↓ 6.2 [2]	-	**No**	-	[54]
	Abn-CBD	U46619	4.6	0.1–100	↑	↑	**-**	-	-	-	**-**	↑	-	-	-	**No**	**No**	↓ 5.4 [2]	-	-	**No**	-	[54]

[1] in the presence of AM251; [2] antagonistic potency (pA_2); [3] statistically significant influence was noticed for virodhamine (30 μm) only; *, used antagonists; ↑, enhancing effect; ↓, weakening effect; **No**, no effect; -, not determined. Abbreviations: 2-AG, 2-arachidonoylglycerol; 2-AGE, noladin ether; 5-HT, serotonin; AEA, anandamide; Abn-CBD, abnormal cannabidiol; APA, apamin, blocker of $K_{Ca}2.3$; AM251, CB_1-R antagonist; AM630, CB_2-R antagonist; CAPS, capsazepine; CB_1-R, cannabinoid receptor type 1; CB_2-R, cannabinoid receptor type 2; CBD, cannabidiol; CHTX, charybdotoxin, $K_{Ca}1.1$ and $K_{Ca}3.1$ inhibitor; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; eCB, historically called endothelial cannabinoid receptor; eNOS, endothelial nitric oxide synthase; EP_4, prostanoid EP_4 receptor; FAAH, fatty acid amide hydrolase; GW9662, 2-chloro-5-nitrobenzanilide, PPAR-γ receptor antagonist; IBX, iberiotoxin, $K_{Ca}1.1$ inhibitor; IP, prostacyclin receptor; INDO, indometacin, COX-1/COX-2 inhibitor; JZL184, monoacylglycerol lipase inhibitor; K_{Ca}, calcium-activated potassium channels; $K_{Ca}2.3$, $K_{Ca}3.1$, $K_{Ca}1.1$, calcium-activated potassium channels with small, intermediate and large conductivity for K^+, respectively; L-NAME, N G-nitro-l-arginine methyl ester, eNOS inhibitor; L-161982, EP_4 receptor antagonist; LPI, L-alpha-lysophosphatidylinositol; MAGL, monoacylglycerol lipase; nimes: nimesulid, COX-2 inhibitor; O-1918, eCB receptor antagonist; pCa 6.3, buffer, containing free Ca^{2+} concentrations of 0.316 μM; pEC_{50}, the negative logarithm of the concentration causing a half-maximum effect; Phe, phenylephrine; PPAR-γ, peroxisome proliferator-activated receptor gamma; RO1138452, IP receptor antagonist; SR141716, rimonabant, CB_1-R antagonist; SR144528, CB_2-R receptor antagonist; TRAM-34, triarylmethane-34, $K_{Ca}2.3$ inhibitor; TRPV1, transient receptor potential vanilloid 1; U46619, prostanoid TP receptor agonist; UCL1684, 6,10-diaza-3(1,3)8,(1,4)-dibenzena-1,5(1,4)-diquinolinacy clodecaphane, $K_{Ca}2.3$ inhibitor; URB597, FAAH inhibitor; VIR, virodhamine.

At the outset, it is worth noting that the most frequently used pulmonary vasoconstrictors (i.e., U46619 (an analogue of TXA_2) and 5-HT (Table 3)) reflect the vasoconstrictors involved in PH's pathophysiology (see Section 4). LPI shows the strongest vasodilatory effect, but this effect could be due to the use of phenylephrine for vasoconstriction [23]. Additionally, CBD and LPI cause a time-dependent relaxation of human pulmonary vessels. Single concentrations of CBD [22] and LPI [23] produce an initial relaxation of the vessels of about 20% after 15 min, increasing to about 70% after 120 min.

In addition to the best-known endocannabinoids, in this paper, we show for the first time that the three endocannabinoid-like molecules, i.e., 2-AGE, ARA-S, and ODA, can cause a slowly developing relaxation of the endothelium-intact human pulmonary arteries (hPAs), with the following rank-order of potencies (according to their pEC_{50} values): AEA (4.8) > 2-AGE (4.6) > ARA-S (4.1) > ODA (<4) (see Figure 1). To date, the vasodilatory effects of ODA [55] and ARA-S [56] have been investigated in rMAs and aortas only. Moreover, 2-AGE was previously shown to relax rabbit pulmonary arteries [52]. 2-AGE may be an interesting focus for future studies on pulmonary arteries since oppositely to unstable 2-AG, 2-AGE does not convert to metabolites with vasoconstrictor activity in rabbit pulmonary circulation [18].

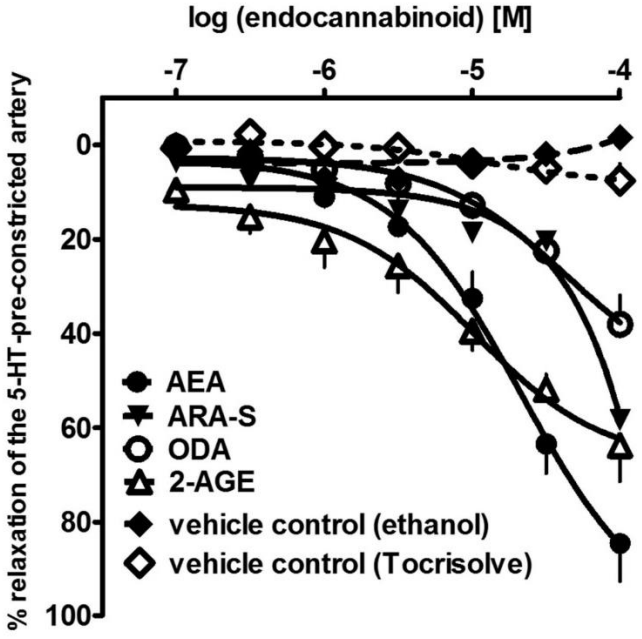

Figure 1. Concentration–response curves of endocannabinoid anandamide (AEA) and endocannabinoid-like molecules: N-arachidonoyl-L-serine (ARA-S), oleamide; cis-9-octadecenoamide (ODA), noladin ether; 2-arachidonyl-glycerol ether (2-AGE) or vehicles for their vasorelaxant effects on endothelium-intact rings of isolated human pulmonary artery. Results are expressed as percentage relaxation of the isometric contraction induced by serotonin (5-HT, 1 μM). Mean ± SEM of 5 tissues is shown for each curve. In few cases, SEM is smaller than or equal to the size of symbols.

Cannabinoids cause multidirectional pulmonary vasodilatory effects mediated by the vascular endothelium and/or the COX-dependent pathway, potassium channels (i.e., calcium-activated potassium channels (K_{Ca}) with small ($K_{Ca}2.3$), intermediate ($K_{Ca}3.1$), and large ($K_{Ca}1.1$) conductance), cannabinoid receptors, and others (see Table 3).

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6. Endothelium-Dependent Mechanisms of Pulmonary Vasorelaxation

In all the studies performed on isolated hPAs and animal pulmonary arteries (Table 3), the removal of the endothelium impairs vascular relaxation. This suggests the contribution of endothelium-dependent mechanisms. The removal of the vascular endothelium reduces the relaxation induced by the highest concentrations of AEA [24] and VIR [53] in hPAs by approximately 65%. The endothelium was also observed to be involved in CBD- [22], 2-AG- [21], LPI- [23], and Abn-CBD-induced relaxation [9] in hPAs. Similarly, in animal studies, the removal of the endothelium attenuates the relaxation induced by 2-AGE in rabbit pulmonary arteries [52] and by AEA or Abn-CBD in rat pulmonary arteries (rPAs) (see Table 3) [54]. In systemic vessels, endothelium denudation modifies the relaxation effect in 70% of the studies published after Stanley et al. (see Table 2) [14]. In summary, regardless of the species and vasoconstricting factors, the vascular endothelium probably plays an important role in pulmonary vasorelaxation. The mechanisms that could account for the endothelium-dependent vasodilating effects are described below (i.e., the arachidonic-acid-derived pathway, K_{Ca} channels, and the involvement of NO (see Table 3)).

6.1. Arachidonic-Acid-Derived Pathway

Several lines of evidence have shown that the endothelium-dependent component of cannabinoid-evoked vasorelaxation may be mediated by arachidonic-acid-derived products that occur as a result of further transformation in the COX-1/COX-2-dependent pathway [57]. The administration of URB597, an FAAH inhibitor, and indomethacin, a non-selective COX-1/COX-2 inhibitor, decreases the relaxation induced by AEA [24] and VIR [53] in hPAs. Indomethacin and nimesulide (a selective COX-2 inhibitor) inhibite the CBD-mediated relaxation of hPAs [22], suggesting the involvement of arachidonic-acid-derived metabolites in relaxation (see Table 3). Similar effects are observed in rPAs, where URB597 and indomethacin were also found to inhibit AEA-induced relaxation [54]. Some of the most important endocannabinoid-related products of the COX-1/2-dependent pathway are PGI_2 and prostaglandins (mainly PGE_2) [58]. PGE_2 exerts dichotomous vascular activities and may cause vasorelaxation via the prostaglandin receptor EP_2 or EP_4 [22,59] and vasoconstriction via the receptor EP_1 or EP_3 [18]. Notably, as a result of the weakened endothelial functions in PH, the concentration of PGI_2 decreases. By binding with its membrane receptor, PGI_2 stimulates adenylate cyclase, which produces cyclic adenosine monophosphate (cAMP), which not only induces relaxation, but also exhibits anti-proliferative properties [60]. The involvement of IP receptors in the AEA-induced relaxation of hPAs [24] and rPAs [54] was also confirmed (Table 3). In addition to the above, it was proposed that CBD-dependent pulmonary vasodilation is mediated by the stimulation of the IP and EP_4 receptors, as antagonists of these receptors were observed to reduce the relaxation effect (see Table 3) [22].

Notably, in an isolated mouse perfused lung model, AEA induces the contraction of the pulmonary vessels through the products of FAAH-induced AEA degradation [20]. The authors showed that AEA does not modulate vascular tone in large isolated pulmonary arteries (preconstricted with phenylephrine). In addition, hypoxia can also increase the levels of an important precursor of vasoconstrictive eicosanoids and AA in pulmonary artery smooth muscle cells (PASMCs). Moreover, the hypoxia-induced elevation of AEA and AA is restricted to PASMCs and does not occur in pulmonary endothelial cells [20]. An increase in PAP under the influence of AEA was shown in an isolated perfused rabbit lung model; the authors suggested that this increase may be related to AEA's degradation into vasoconstricting metabolites [18], as COX-1/2-dependent-pathway metabolites might also possess vasoconstriction potency. However, more research is necessary to conclusively determine why AEA presents completely different effects between isolated vessels and the perfused lung model.

6.2. Vasorelaxation's Dependence on Calcium-Dependent Potassium Channels

K_{Ca} are important in regulating pulmonary vascular tone, and impaired K_{Ca} function can lead to PH [61]. The ability of high KCl concentrations to abolish or reduce the vasorelaxation induced by cannabinoids, including AEA [24,54], VIR [53], CBD [22], and Abn-CBD [9,54], suggests the direct or indirect involvement of potassium channels (including K_{Ca}). Charybdotoxin and apamin, which are $K_{Ca}1.1$ and $K_{Ca}3.1$ or $K_{Ca}2.3$ inhibitors, respectively, reduce the vasorelaxant effects of Abn-CBD [9] and VIR in hPAs [53] (Table 3). This reduction may be related to the involvement of endothelium-dependent hyperpolarization (EDH) [62], which is sensitive to the combined administration of apamin and charybdotoxin in the pulmonary vasorelaxation mechanism. Iberiotoxin, which is an inhibitor of $K_{Ca}1.1$ channels, reduces AEA-induced vasorelaxation in the human pulmonary vascular bed [24]. Similarly, iberiotoxin and TRAM-34, which are inhibitors of $K_{Ca}1.1$ and $K_{Ca}3.1$, respectively, significantly reduce the CBD-induced relaxation of hPA (see Table 3) [22].

In addition to the above-mentioned K_{Ca}, the expression of the two-pore-domain potassium (K2P) channel was confirmed in rat and human PASMCs [63]. AEA attenuates hypoxia-induced vasoconstriction (which is one of the pathogenetic factors in PH) via the inhibition of the K2P channel in murine intra-acinar and pre-acinar arteries and does not change the vascular calibre under normoxia [63].

6.3. Regulation of Pulmonary Vascular Tension by NO

The incubation of isolated human pulmonary vessels with N^G-nitro-l-arginine methyl ester (L-NAME), an endothelial nitric oxide synthase (eNOS) inhibitor, reduces the relaxation induced by AEA [24] and, to a lesser extent, that induced by VIR [53]. A similar effect was observed for AEA in the rPA [54] (see Table 3). Notably, the NO-dependent component of AEA-evoked relaxation may be the result of direct or indirect interactions with PPAR-γ, which stimulates NO production and potentiates NO's bioavailability [64]. In contrast to the above, it was previously shown that NO does not participate in the vasorelaxation induced by exogenous cannabinoids, especially that induced by stable analogues such as Abn-CBD [9]. Similarly, CBD-induced hPA relaxation is also NO-independent [22]. In systemic vessels, NO appears to be involved in the AEA- [38] and CBD-induced [37] relaxation of human mesenteric arteries (hMAs) (Table 2). Similarly, NAGly- [39], CBD- [46], JHW-133-, and APCA-induced [41] relaxation in rMAs was shown to be attenuated by L-NAME administration. In hMAs, L-NAME was shown to attenuate the vasodilatory effects mediated by CBD, and CBD was found to increase eNOS phosphorylation in human endothelial cells [37]. No evidence indicated the involvement of NO in Abn-CBD-mediated relaxation in rat retinal capillaries (see Table 2). These discrepancies in the mechanism of action may be due to differences between the species and structures/properties in different cannabinoid groups (endocannabinoids, phytocannabinoids, and synthetic cannabinoids such as Abn-CBD).

7. Receptor-Mediated Vasodilatation

It was previously suggested that the mechanisms inducing the relaxation of pulmonary vessels under the influence of cannabinoids include CB_1-Rs/CB_2-Rs [21], other CB receptors such as eCB-Rs [9,37,52–54], the cannabinoid-receptor-related orphan G-protein-coupled receptors GPR55 and GPR18 [23], and the non-cannabinoid receptors PPAR-γ [22,23], TRPV1, and TRPV4 [22]. This argument is reinforced by the fact that the presence of these receptors in the endothelium and/or smooth muscle cells was confirmed (Table 1).

7.1. Mechanism Dependent on CB_1-Rs and CB_2-Rs

The administration of the CB_1-R antagonist AM251 and/or rimonabant attenuates 2-AG-mediated relaxation in hPAs [21], 2-AGE, and Abn-CBD-mediated relaxation in rabbit pulmonary arteries [52], suggesting the involvement of these receptors in vasodilation (see Table 3). CB_1-R antagonists do not affect the AEA-induced relaxation of hPAs [24] or

the AEA- [54], CBD- [22], and Abn-CBD-induced [54] relaxation of rPAs. Moreover, the administration of rimonabant at a concentration of 100 nM does not reduce VIR-induced relaxation, which excludes the participation of CB_1-Rs. However, this effect was observed at a concentration of 5 μM; however, a higher concentration of rimonabant antagonizes eCB-Rs (see Table 3) [53]. Additionally, WIN 55,212-2, a synthetic agonist of CB_1-Rs and CB_2-Rs, does not induce the relaxation of pulmonary vessels [53].

There are indications of a previously unknown CB_1-R-dependent endocannabinoid-mediated potential protective mechanism against excessive vasoconstriction (mainly mediated by 2-AG). AM251 attenuates 2-AG-induced vasorelaxation, indicating the involvement of CB_1-Rs in the relaxation mechanism (see Table 3) [21]. It was suggested that vasoconstrictors such as TXA_2 and ANG II stimulate the $G_{q/11}$ protein, stimulating the release of 2-AG from the vascular endothelium. By acting on CB_1-Rs, 2-AG produces vasodilation in hPAs [21,48], which may play a protective role against excessive increases in pressure in the pulmonary circulation through a so-called negative-feedback mechanism. The administration of JZL184, a MAGL inhibitor, enhances the relaxant effects of 2-AG in hPAs, suggesting that the vasorelaxant effect is caused by undegraded 2-AG, not the metabolites of 2-AG. Moreover, this experiment further confirmed that 2-AG, not AEA, is responsible for this effect, because, as described above, AEA does not act through CB_1-Rs (see Table 3). In addition, contractions induced by U46619 in hPAs with preserved endothelium are enhanced by the presence of the DAGL inhibitor RHC80267 (responsible for the formation of 2-AG). This effect was not observed in pulmonary arteries with the endothelium removed. Experiments with RHC80267 suggested that the rapid, contractile-stimulated synthesis of 2-AG and its release from endothelial cells plays a protective role [21].

Conversely, in systemic vessels, CB_1-Rs are involved in AEA- [38], CBD- [22,37], Meth-AEA- [42,47], and ACPA-induced relaxation [41] in human and animal mesenteric arteries (see Table 2). In systemic circulation, however, it has not yet been confirmed that the mechanism underlying the 2-AG-induced relaxation of hMAs depends on CB_1-Rs. Moreover, it was suggested that this effect is exerted by metabolites resulting from the degradation of 2-AG in the COX-1-dependent pathway [14].

Notably, to date, no studies have confirmed the role of CB_2-Rs in the cannabinoid-induced relaxation of isolated pulmonary vessels (Table 3) [22,24,53,54]. However, Zoratti et al. [65] demonstrated the presence of CB_2-Rs in a calf pulmonary artery endothelial (CPAE) cell line; these CB_2-Rs were found to be 86% homologous to the corresponding regions of the human CB_2-R sequences. The functional analysis also showed that AEA initiates Ca^{2+} signalling in CPAE cells through the CB_2-R activation. Although it is generally accepted that CB_2-Rs are not directly involved in vascular relaxation [37], it was previously reported that the administration of the CB_2-R inhibitor AM630 reduces the CBD-induced vasorelaxant effect in rat femoral arteries [45]. However, because CBD does not direclty activate CB_2-Rs, the specific mechanism of action is unknown; it was suggested that CBD changes the function of this receptor.

7.2. Other G-Protein-Dependent Receptors

Previously, a cannabinoid endothelial receptor sensitive to O-1918 was considered to be a site of action for vasorelaxation. However, since this putative receptor has not yet been cloned, it remains uncertain whether it can truly be classified as a receptor. It was observed that the administration of the eCB-R antagonist O-1918 [9,53,54] reduces the AEA- [24] and VIR-induced [53], but not CBD-induced, relaxation of hPAs (see Table 3) [22]. Interestingly, three independent studies exploring the effects of Abn-CBD on the pulmonary vessels of humans [9], rabbits [52], and rats [54] suggested that this relaxation effect may depend on the presumed eCB-R, because the administration of O-1918 impairs relaxation. The administration of the pertussis toxin (PTX) (400 ng/mL, for 2 h) partially inhibits the Abn-CBD-induced vasodilation of endothelium-intact human arteries, which confirmed the involvement of a G_i/G_o-coupled eCB-Rs [9]. These differences in the vascular mechanisms of action of CBD and Abn-CBD, coupled with the fact that CBD is a partial

agonist/antagonist of GPR18 while Abn-CBD is an agonist of GPR18, suggest that the unclassified eCB-R is probably GPR18 [15,29]. However, the putative eCB receptor antagonist may act independently of the G-protein-coupled receptors (GPCRs). Additionally, this receptor influences the functional properties of many ion channels and transporters located in the vascular system [15,66]. Reports that O-1918, after endothelial removal, also attenuates the vasodilatory effect suggest that O-1918's site of action may be in the vascular smooth muscle [67]. Additionally, it was shown that O-1918 is an inhibitor of the Na^+/Ca^{2+} exchanger [68] and inhibits the activity of $K_{Ca}1.1$ channels [69], which may contribute to the regulation of vascular tone. However, this issue has not yet been unequivocally resolved in the literature [15,29,66,70].

Another receptor with confirmed expression in hPAs is GPR55, which exhibits vasorelaxant properties in the above-mentioned arteries (Table 1). LPI, an endogenous non-cannabinoid agonist of the GPR55 receptors, depending on concentration and time, causes the relaxation of isolated hPAs. The participation of GPR55 receptors in functional studies was confirmed by the use of their antagonist CID16020046, which significantly reduces the relaxation responses of hPAs stimulated with LPI [23].

7.3. Other G-Protein-Independent Receptors

In the pulmonary vessels, according to the current literature, only CBD causes relaxation dependent on TRPV1 receptors (see Table 3) [22]. Importantly, the presence of the TRPV1 receptors in human pulmonary vessels was confirmed (see Table 1) [22]. Capsazepine, an antagonist of TRPV1 receptors, was not observed to reduce the rPA [54] and hPA [24,53] relaxation induced by endocannabinoids such as AEA and VIR (Table 3).

The role of TRPV1 receptors in PH is unclear. Zhang et al. [71] suggested that, on the one hand, TRPV1 induces an increase in intracellular calcium ($[Ca^{2+}]_i$) in PASMCs and can cause vascular contractions, as well as promoting smooth muscle cell proliferation, which can lead to PH. On the other hand, the activation of TRPV1 in sensory nerves can release neuropeptides, including the calcitonin-gene-related peptide (CGRP) [71]. CGRP causes the relaxation of blood vessels and inhibits their proliferation, which may be beneficial in PH [71]. Moreover, pre-treatment with capsaicin, a specific activator of TRPV1, was found to reverse PH by alleviating inflammation [72]. Thus, the potential role of TRPV1 in PH should be further investigated.

The presence of TRPV4 receptors and their involvement in vascular relaxation mechanisms was confirmed by Addison et al. [73]. The pharmacological activation of TRPV4 receptors with the selective agonist GSK1016790A results in the relaxation of endothelium-intact rPAs preconstricted with phenylephrine [73]. In addition, the TRPV4-receptor antagonist HC067047 reduces the vasodilatory response to GSK1016790A [73,74]. Despite the above, it has not been confirmed that TRPV4 receptors participate in CBD-induced relaxation since the administration of RN1734, which antagonizes the TRPV4 receptors, does not affect relaxation [22]. Conversely, Ho et al. [36] confirmed that these receptors participate in the rMA relaxation induced by 2-AG through two antagonists, HC067047 and RN1734 (see Table 2). Moreover, TRPV4 receptors are involved in the proliferation and migration of PASMCs and may serve as a crucial target in the treatment of PH [75].

Recently, research has suggested the potential benefits of stimulating PPAR-γ receptors to alleviate PH. The PPAR-γ antagonist GW9662 reduces the time-dependent relaxation of hPAs induced by CBD (10 μM) (see Table 3) [22]. Previous studies on the potential beneficial effects of PPAR-γ receptor agonists demonstrated the PPAR-γ-receptor-mediated relaxation of human pulmonary vessels preconstricted with U46619 [76]. In addition to the above, PPAR-γ agonists exert beneficial effects on pulmonary vascular remodelling and lung morphology. Indirect evidence for the utility of PPAR-γ agonists in the treatment of PH lies in the fact that the deletion of this receptor in mouse smooth muscle [77] and endothelial cells caused the hypertrophy of the small distal pulmonary arteries and, consequently, induced PH [78]. PPAR-γ ligands interfere with the production of matrix metalloproteinases that can be activated by elastase, which was shown to prevent and

reverse PH in rats. In addition to the above, PPAR-γ has anti-inflammatory properties, which include the suppression of factors related to PH, such as interleukin-6 (IL-6) and monocyte chemoattractant protein (MCP-1). PPAR-γ also protects endothelial cells against apoptosis [79]. PPAR-γ expression was found to be reduced in patients with primary and secondary PH [80], and hypoxia was found to reduce PPAR-γ expression in human pulmonary vessels [81,82]. CBD is a functional PPAR-γ agonist and was observed to cause the time-dependent relaxation of rat aortas. This effect is inhibited by the PPAR-γ antagonist GW9662, which confirms the effect of PPAR-γ on aortic relaxation [83].

8. Cannabinoids in PH—In Vivo and In Vitro Studies

Although the effects of cannabinoids on isolated vessels have been fairly well researched, there are still very few in vivo studies. In this review, the terms "PAH" and "PH" are reserved for human and experimental conditions, respectively [84,85]. An interesting look at the use of cannabinoids in PH therapy was presented in the latest study on CBD administration in an animal model of PH [19]. This PH model is induced in 6-to-8-week-old rats via the subcutaneous administration of 60 mg/kg MCT. The use of MCT to create an experimental model allows for the relatively simple mapping of PH in the human population by selectively damaging pulmonary vessels without adversely affecting systemic blood vessels [84]. The chronic administration of CBD as a prophylactic (see Table 4) improves blood oxygen saturation and lowers right ventricular systolic pressure (RVSP) without impacting systemic BP. CBD also reduces pulmonary arterial hypertrophy by about 30%, without any effects on RV hypertrophy [19], and normalized the plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA). This effect is beneficial, because the levels of PAI-1 and t-PA are increased in PH. The above changes may partly correlate with increases in endogenous cannabinoid concentrations and AEA and NAGly in CBD-treated animals [19] (Table 4), because both endocannabinoids can relax the pulmonary [9,24,54] and systemic vessels [38,39,59] (see Tables 2 and 3). Importantly, the chronic administration of the same dose of CBD does not change the BP or heart rate (HR) in spontaneously hypertensive rats (SHRs), rats with secondary hypertension induced by deoxycorticosterone acetate salt (DOCA salt), or their controls with normal pressure [86].

Lu et al. [87] also suggested the potential benefits of using CBD (in preventive and therapeutic models; see Table 4) in PH treatment and showed that CBD, in a preventive model, is more effective in decreasing PH phenotypes in PH mice. Mice (in a sugen-hypoxia-induced PH model) and rats (in an MCT-induced PH model) treated with CBD present lower RVSP and reduce RV and pulmonary-artery hyperproliferation. CBD also reduces the mRNA levels of inflammatory mediators such as IL-6 and tumour necrosis factor-α (TNF-α) in mouse lung tissue (see Table 4) [87]. Moreover, CBD (10 μM) was shown to inhibit the hyperproliferation of mouse PASMCs without any harmful effects on normal PASMCs. CBD was also found to recover dysfunctional mitochondria under conditions of hypoxia and relieve oxidative stress in human and mouse PASMC cell cultures. The effectiveness of CBD was also compared to that of drugs commonly used for PH, and the results suggest that CBD is as effective as bosentan or beraprost [87].

Other studies have shown an increase in PAP after the administration of AEA and 2-AG in isolated, ventilated, and buffer-perfused rabbit lungs. 2-AG showed more pronounced effects at lower concentrations. Anandamide presents a similar relationship, and an increase in PAP was observed, depending on the dose of AEA (Table 4). The authors suggested that the products from the breakdown of endocannabinoids are further metabolized to PGE_2 and TXA_2 (via COX-2), with vasoconstriction properties, in pulmonary arteries [18]. A similar theory was presented by Wenzel et al. [20]. According to the authors, AEA is a mediator of HPV via FAAH-dependent metabolites and is involved in the generation of PH, as discussed above.

Table 4. Influence of cannabinoids on pulmonary circulation in in vivo or in vitro studies.

Species	Model	Cannabinoid	Dose/Concentration/Route of Administration	Effect	References
rabbit	isolated, ventilated, and buffer-perfused lung	AEA	0.5–5 μM	↑ pulmonary arterial pressure	[18]
		2-AG	0.2–0.4 μM		
rat	MCT-induced PH (60 mg/kg)	CBD	10 mg/kg for 21 days, preventive model, i.p.	↓ RVSP ↓ pulmonary arterial hypertrophy **No** right ventricular hypertrophy ↑ blood oxygen saturation ↑ concentration of endogenous cannabinoids in lung tissue: AEA, 2-LG, LEA, POEA, EPEA and NAGly ↓ the plasma concentrations of PAI-1 and t-PA	[19]
				↓ RVSP ↓ pulmonary arterial hypertrophy ↓ right ventricular hypertrophy	[87]
mouse	sugen-hypoxia-induced PH	CBD	10 mg/kg for 21 days, preventive model, i.g.	↓ RVSP ↓ pulmonary arterial hypertrophy ↓ right ventricular hypertrophy ↓ mRNA levels of IL-6 and TNF-α in lung tissue	[87]
			10 mg/kg for 14 days after PH induction, therapeutic model i.g.	↓ RVSP ↓ pulmonary arterial hypertrophy ↓ right ventricular hypertrophy	
	PH-PASMC		10 μM for 2 h	↓ hyperproliferation ↓ mRNA levels of chemokine CCL2 and CXCL10 ↓ oxidative stress in mitochondria	
human	hypoxia induced HPASMC cell culture	CBD	10 μM for 2 h and 12 h	recover the dysfunctional mitochondria in hypoxia condition: ↓ oxidative stress ↓ excessive glycolysis	[87]

↑, increase; ↓, decrease; **No**, no change. Abbreviations: 2-AG, 2-arachidonoylglycerol; 2-LG, linoleoylglycerol; AEA, anandamide; CBD, cannabidiol; CCL2, monocyte chemoattractant protein-1; CXCL10, chemokine (C-X-C motif) ligand 10; EPEA, eicosapentaenoyl ethanolamide; i.g., intragastric administration; IL-6, interleukin-6; i.p., intraperitoneal injections; LEA, linolenoyl ethanolamide; NAGLy, N-arachidonoyl glycine; PH, pulmonary hypertension; PAI-1, plasminogen activator inhibitor-1; PASMCs, pulmonary artery smooth muscle cells; POEA, palmitoleoyl ethanolamide; RVSP, right ventricular systolic pressure; TNF-α, tumour necrosis factor-alpha; tPA, tissue plasminogen activator.

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RV failure is undoubtedly one of the worst consequences of PH. Duerr et al. [88] suggested that the ECS may play an important role in PH related to the endocannabinoid–CB_2-R axis. In a mouse PH model induced by left pulmonary artery occlusion, researchers found that CB_2-R-deficient ($Cnr2^{-/-}$) mice had stronger cardiomyocytic hypertrophy and an increased Fulton's index. The above-described effects of cannabinoids on pulmonary vascular tone and new reports on the potential beneficial effect of CBD on the animal model of PH may provide a foundation for further research. Among cannabinoids, it may be useful to explore new therapeutics for PH, especially when it is possible to create synthetic cannabinoids with selective and more concentrated actions.

9. Conclusions

(Endo)cannabinoids play a role in regulating pulmonary vascular tone through endothelium-dependent and/or receptor-based mechanisms (Figure 2), which may contribute to decreasing pulmonary resistance. Moreover, the endocannabinoid negative-feedback mechanism in pulmonary arteries was found to be responsible for attenuating agonist-induced vasoconstriction, which may also play an important role in the treatment of PH. CBD, which was approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of drug-resistant seizures and spasticity in adult patients with multiple sclerosis, also exerts a protective effect on the vascular endothelium, decreases RVSP and/or heart remodelling and increases saturation in experimental PH, in addition to its vasorelaxant effects on pulmonary arteries. Therefore, (endo)cannabinoids represent a potential new treatment strategy as an add-on therapy for PH. Nevertheless, it should be emphasized that no clinical trials with cannabinoids in PH have yet been conducted; thus, their therapeutic potential has not been yet translated into clinical practice. In addition, single experimental studies showed that AEA and 2-AG can contract vessels and/or increase PAP. Further research, both experimental and clinical, is needed to explain these inaccuracies.

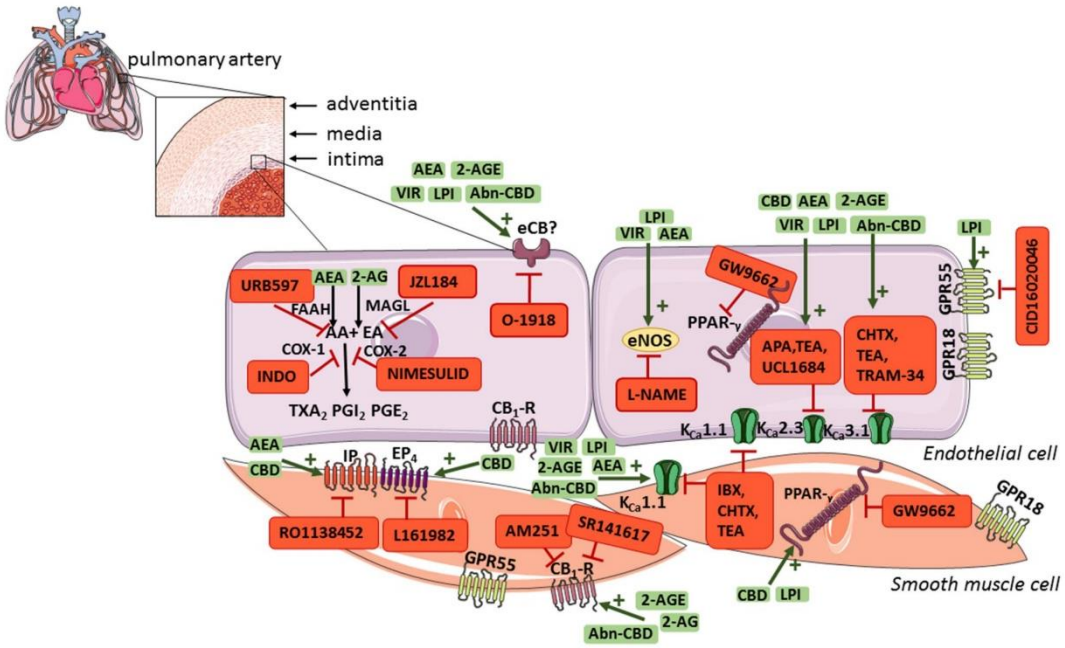

Figure 2. The location of the endocannabinoid system components and potential mechanisms involved in the cannabinoid-induced vasorelaxation in pulmonary arteries. Abbreviations: 2-AG, 2-arachidonoylglycerol; 2-AGE, noladin ether; AA, arachidonic acid; AEA, anandamide; Abn-CBD, abnormal cannabidiol; APA, apamin, $K_{Ca}2.3$ inhibitor; AM251, CB_1-R

antagonist; AM630, CB_2-R antagonist; CB_1-R, cannabinoid receptor type 1; CB_2-R, cannabinoid receptor type 2; CBD, cannabidiol; CHTX, charybdotoxin, $K_{Ca}1.1$ and $K_{Ca}3.1$ inhibitor; CID16020046, GPR55 receptor antagonist; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; EA, ethanolamine; eCB, historically called endothelial cannabinoid receptor; eNOS, endothelial nitric oxide synthase; EP_4, prostanoid EP4 receptor; FAAH, fatty acid amide hydrolase; GPR18, G protein-coupled receptor 18; GPR55, G-protein-coupled receptor 55; GW9662, PPAR-γ receptor antagonist; IBX, iberiotoxin, $K_{Ca}1.1$ inhibitor; IP, prostacyclin receptor; INDO, indometacin, COX-1/COX-2 inhibitor; JZL184, monoacylglycerol lipase inhibitor; $K_{Ca}2.3$, $K_{Ca}3.1$, and $K_{Ca}1.1$, calcium-activated potassium channels with small, intermediate, and large conductivity for K^+, respectively; LPI, l-alpha-lysophosphatidylinositol; L-NAME, N G-nitro-l-arginine methyl ester, eNOS inhibitor; L161982, EP_4 receptor antagonist; K_{ca}, calcium-activated potassium channels; MAGL, monoacylglycerol lipase; nimesulid, COX-2 inhibitor; O-1918, eCB receptor antagonist; pEC_{50}, the negative logarithm of the concentration causing a half-maximum effect; PGE_2, prostaglandin E2; PGI_2, prostacyclin; PPAR-γ, peroxisome proliferator-activated receptor-gamma; RO1138452, IP receptor antagonist; SR141716, rimonabant, CB_1-R antagonist; SR144528, CB_2-R antagonist; TEA, tetraethylammonium, $K_{Ca}2.3$ and $K_{Ca}3.1$ inhibitor; TRAM-34, triarylmethane-34, $K_{Ca}2.3$ inhibitor; TRPV1, transient receptor potential vanilloid 1; TXA_2, thromboxane A2; UCL1684, 6,10-diaza-3(1,3)8,(1,4)-dibenzena-1,5(1,4)-diquinolinacy clodecaphane, $K_{Ca}2.3$ inhibitor; URB597, FAAH inhibitor; VIR, virodhamine. This figure was prepared using a template on the Servier Medical Art website.

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Abbreviations

2-AGE	noladin ether
Abn-CBD	abnormal-cannabidiol
APCA	arachidonylcyclopropylamide
ARA-S	arachidonoyl-L-serine
$CB_{1,2}$-R	cannabinoid receptor types 1 and 2
CBD	cannabidiol
CBRs	cannabinoid receptors
DAGL-α,β	diacylglycerol lipases α,β
DHEA	docosahexaenoyl ethanolamid
DEA	docosatetraenoyl ethanolamide
ECS	endocannabinoid system
EDH	endothelium-dependent hyperpolarization
EPEA	eicosapentaenoyl ethanolamide
ERAs	endothelin receptor antagonists
HEA	homo-γ-linolenyl ethanolamide
hPAs	human pulmonary arteries
LEA	inolenoyl ethanolamide
2-LG	linoleoylglycerol
LPI L	alpha-Lysophosphatidylinositol
MCT	monocrotaline
MethAEA	methanandamide
NAGly N	arachidonoyl glycine
ODA	oleamide
OEA	oleoyl ethanolamide
PAH	pulmonary arterial hypertension
POEA	palmitoleoyl ethanolamide

PAP	pulmonary arterial pressure
PASMCs	pulmonary artery smooth muscle cells
PEA	ethanolamide
PH	pulmonary hypertension
PPAR-γ	peroxisome proliferator-activated receptor-γ
PVR	pulmonary vascular resistance
rPA	rat pulmonary artery
RVSP	right ventricular systolic pressure
SEA	stearoyl ethanolamide
U46619	analogue of thromboxane A2
VIR	virodhamine

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Rozdział 11. Pierwsza praca oryginalna

molecules

MDPI

Article

Cannabidiol Improves Antioxidant Capacity and Reduces Inflammation in the Lungs of Rats with Monocrotaline-Induced Pulmonary Hypertension

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Abstract: Cannabidiol (CBD) is a plant-derived compound with antioxidant and anti-inflammatory properties. Pulmonary hypertension (PH) is still an incurable disease. CBD has been suggested to ameliorate monocrotaline (MCT)-induced PH, including reduction in right ventricular systolic pressure (RVSP), a vasorelaxant effect on pulmonary arteries and a decrease in the white blood cell count. The aim of our study was to investigate the effect of chronic administration of CBD (10 mg/kg daily for 21 days) on the parameters of oxidative stress and inflammation in the lungs of rats with MCT-induced PH. In MCT-induced PH, we found a decrease in total antioxidant capacity (TAC) and glutathione level (GSH), an increase in inflammatory parameters, e.g., tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), nuclear factor kappa B (NF-κB), monocyte chemoattractant protein-1 (MCP-1), and cluster of differentiation 68 (CD68), and the overexpression of cannabinoid receptors type 1 and 2 (CB_1-Rs, CB_2-Rs). Administration of CBD increased TAC and GSH concentrations, glutathione reductase (GSR) activity, and decreased CB_1-Rs expression and levels of inflammatory mediators such as TNF-α, IL -1β, NF-κB, MCP-1 and CD68. In conclusion, CBD has antioxidant and anti-inflammatory effects in MCT-induced PH. CBD may act as an adjuvant therapy for PH, but further detailed preclinical and clinical studies are recommended to confirm our promising results.

Keywords: pulmonary hypertension; cannabidiol; oxidative stress; inflammation; monocrotaline

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1. Introduction

Pulmonary hypertension (PH) is an incurable disease with rapid progress and poor prognosis. PH is defined as when the mean value of pulmonary artery pressure measured by right ventricular catheterization at rest is over 25 mmHg, although it has been recently suggested to reduce this value to 20 mmHg [1]. The pathogenesis of PH is complex, and includes dysfunction of the vascular endothelium accompanied by excessive vasoconstriction, increased oxidative stress, enhanced inflammation, remodeling of pulmonary arteries with intimal hypertrophy, and right heart failure, which in consequence leads to premature death. Increased oxidative stress and inflammation induce the remodeling of pulmonary vessels, and participate in vasoconstriction in addition to an increase in pulmonary vascular resistance [1,2]. In human PH, perivascular inflammation is associated with an increase in inflammatory mediators (tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), cluster of differentiation 68 (CD68) and nuclear factor kappa B (NF-κB)), in addition to the accumulation and infiltration of inflammatory cells, e.g., macrophages which correlates

with disease progression [3,4]. According to the reports that combination therapy reduces the risk of clinical deterioration of patients, the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) recommend polypharmacology, i.e., use of drugs (or combinations of drugs) that have multiple effects, for PH treatment [5]. Thus, drugs with additional target points, including antioxidant and anti-inflammatory effects are recommended, in order to have a pleiotropic effect on the pathogenesis of PH. The available therapies for PH only improve life quality of patients, but do not guarantee a full recovery, which underscores the need for novel therapeutic strategies [1].

Cannabidiol (CBD) is a non-intoxicating compound isolated from *Cannabis sativa var. indica*. CBD has pleiotropic properties, including those which are antioxidant and anti-inflammatory [6,7]. In studies carried out on the mouse model of oxygen-glucose deprivation/reperfusion injury, it was found that CBD supplementation reduced the level of malondialdehyde, which is a product of lipid peroxidation. Chronic administration of CBD to rats and mice reduced the number of oxidative stress markers, causing a pronounced increase in glutathione (GSH) levels in both animal models—for a review, see Atalay [7]. CBD reduced proinflammatory cytokines in lung tissue in a mouse asthma model [8], and in mouse lipopolysaccharide (LPS)-induced acute lung injuries [9].

CBD is believed to have a weak affinity for cannabinoid receptors type 1 and 2 (CB_1-R and CB_2-R), but may also act indirectly by regulating the levels of certain endocannabinoids [6]. Additionally, CBD is an allosteric negative modulator of the CB_1-R, which means that it could reduce the potency of CB_1-R ligands, thus limiting the adverse effects associated with the activation of CB_1-Rs [6]. Moreover, CBD is able to antagonize the effects of the CB_1-R agonist (CP55940) at nanomolar concentrations, i.e., lower than that resulting from its affinity for these receptors. Additionally, the authors suggest that this antagonism is non-competitive in nature [10]. Moreover, activation of CB_1-Rs has been shown to increase proinflammatory signaling and evoke oxidative stress [11]. Therefore, blocking CB_1-Rs would result in reduction in inflammation and fibrosis in mouse lungs [11]. Activation of CB_2-Rs produces opposite effects, i.e., reduces lung inflammatory damage and pulmonary edema [12], and may be a promising target point in inhibition of inflammation in COVID-19 [11].

Cannabinoids, including CBD, have potentially beneficial effects in PH [13]. CBD has been suggested as an adjuvant therapy for the treatment of PH as a result of its relaxing effect in human pulmonary arteries [14]. In addition, CBD reduced right ventricular systolic pressure (RVSP) and pulmonary vascular hypertrophy in both the rat model of PH induced by monocrotaline (MCT) [15,16], and in the Sugen-hypoxia PH mouse model [16], which suggest that CBD may be able to alleviate PH.

Due to the current trend of using polypharmacological approaches in PH treatment, together with the proven beneficial effects of CBD in various PH models as a consequence of its antioxidant and anti-inflammatory properties, the aim of the study was to evaluate the effect of CBD on the selected parameters of oxidative stress and inflammation in the lungs of rats with MCT-induced PH. Although previous studies suggest that CBD may have anti-inflammatory potential in the mouse model of Sugen-hypoxia-induced PH [16], it has been recently reported that the Sugen-hypoxia model is not preferable in mice, since they display very modest vascular remodeling compared to rats [17–19]. In this respect, both pulmonary vascular remodeling and PH are reversible, and no characteristic perivascular infiltration of monocytes/macrophages is observed [17–19]. The MCT-induced PH model in rats is more recommended because it particularly shows the role of inflammation in the development of PH, and allows the development of damage to pulmonary vessel endothelia with the characteristic perivasculitis [18].

Altogether, we have shown for the first time that CBD improved antioxidant capacity and reduced inflammation in the rat model of MCT-induced PH.

2. Results

2.1. Influence of PH and Chronic Administration of CBD on RVSP

As described previously [15], the RVSP was higher in the MCT group (43.7 ± 3.9 mmHg, $n = 10$) compared to the control (CTR) group (20.03 ± 0.9 mmHg, $n = 10$; $p < 0.001$). Chronic CBD administration reduced the RVSP in the MCT + CBD group (28.2 ± 0.7 mmHg, $n = 10$, $p < 0.001$) compared to MCT group but not in the control group (CTR + CBD 21.4 ± 0.9 mmHg, $n = 10$).

2.2. Influence of PH and Chronic Administration of CBD on Oxidative Stress in Lung Tissue

MCT administration reduced total antioxidant capacity (TAC) (−35%) (Figure 1A) and concentration of GSH (−48%) (Figure 1C) in the lung tissues compared to the CTR group. MCT increased the concentration of 4-hydroxyhexenal (4-HNE) (+44%), which is a product of lipid peroxidation, compared to CTR (Figure 1B). However, MCT-treated rats did not modify the activity of antioxidant enzymes, such as glutathione-disulfide reductase (GSR) and glutathione peroxidase (GPx) (Figures 1D and 1E, respectively).

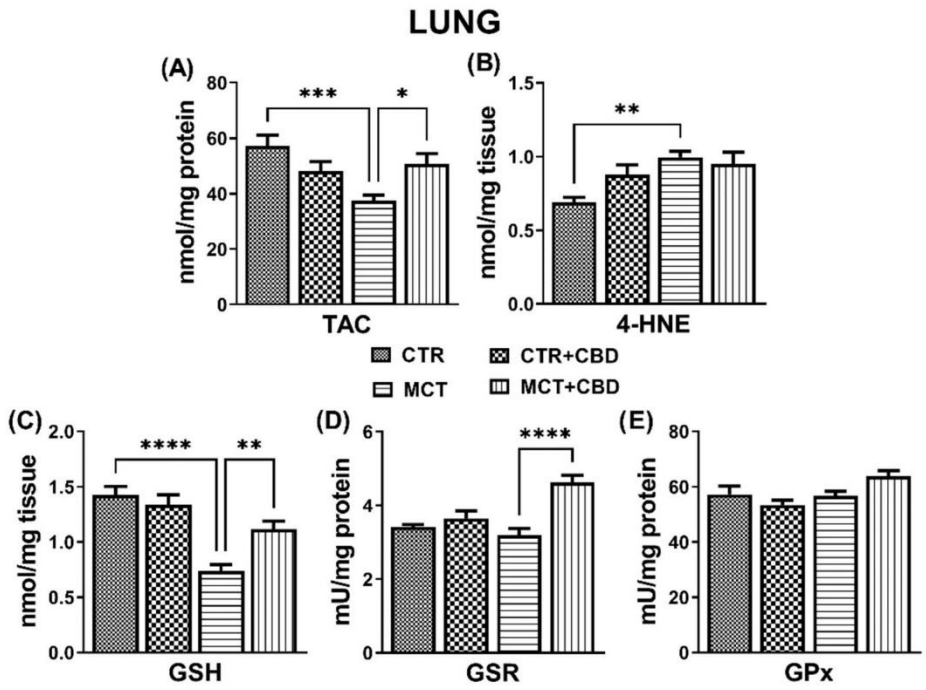

Figure 1. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on redox balance parameters in rats' lungs. Total antioxidant capacity, TAC (**A**); 4-hydroxyhexenal, 4-HNE (**B**); reduced glutathione, GSH (**C**); glutathione-disulfide reductase, GSR (**D**); glutathione peroxidase, GPx (**E**). CBD 10 mg/kg or its vehicle were injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 5–7 per group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, compared to the respective group.

CBD administration to MCT-treated rats increased concentrations in antioxidant compounds TAC (+36%) (Figure 1A) and GSH (+51%) (Figure 1C). CBD also increased the enzymatic activity of GSR (+45%) (Figure 1D) in the MCT + CBD group compared to MCT group. Administration of CBD did not change the MCT-stimulated increased concentration in 4-HNE (Figure 1B), and did not change GPx activity (Figure 1E). No changes of

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the above-mentioned parameters were observed in the lungs of CTR rats receiving CBD (CTR + CBD) (Figure 1).

2.3. Influence of PH and Chronic Administration of CBD on Inflammation Parameters in Lung Tissue

The concentrations of inflammatory parameters NF-κB, TNF-α and monocyte chemoattractant protein-1 (MCP-1) in lung tissues were analysed using an ELISA method. MCT administration increased the concentrations in NF-κB (+100%) (Figure 2A), TNF-α (+67%) (Figure 2B), and MCP-1 (+10-fold) (Figure 2D) in the lung tissues compared to CTR rats.

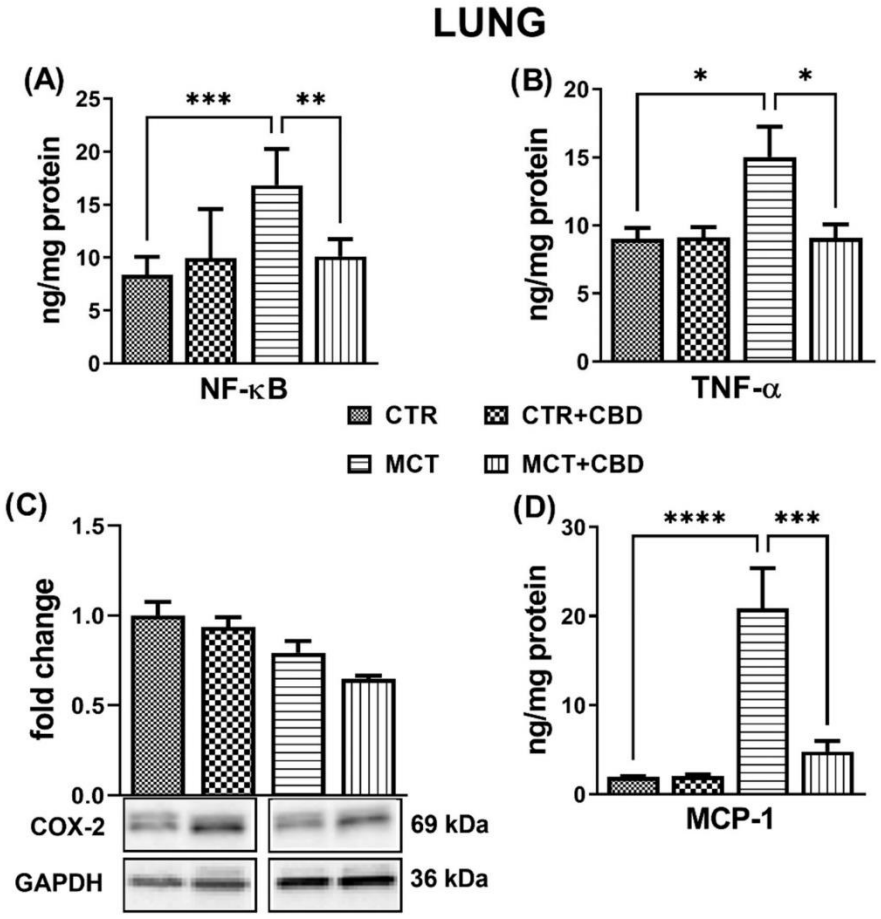

Figure 2. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on parameters of inflammation in rats' lungs. Nuclear factor kappa B, NF-κB (p65) (**A**); tumour necrosis factor alpha, TNF-α (**B**); cyclooxygenase 2, COX-2 (**C**); monocyte chemoattractant protein-1, MCP-1 (**D**). Bar graph C illustrates the fold changes (for the relative fold change in expression in comparison to the respective CTR, whose expression level was set to 1) in the density of COX-2. CBD 10 mg/kg or its vehicle were injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 6–7 per group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, compared to the respective group.

Chronic administration of CBD to MCT-treated rats decreased the concentrations in NF-κB (−40%) (Figure 2A), TNF-α (−40%) (Figure 2B), and MCP-1 (−77%) (Figure 2D). No changes were observed when CBD was administered to CTR rats (Figure 2). The densities of cyclooxygenase 2 (COX-2) evaluated by Western blot method were similar in each group (Figure 2C).

Positive immunohistochemical reaction demonstrated that IL-1β was found mainly in lung macrophages and lung interstitial cells (Figure 3A). A much stronger immunoreactivity of IL-1β (by about 13-fold) was demonstrated in the lungs of MCT rats (Figure 3A,B). MCT administration increased the concentration in IL-1β by about 128% evaluated by an ELISA method (Figure 3C).

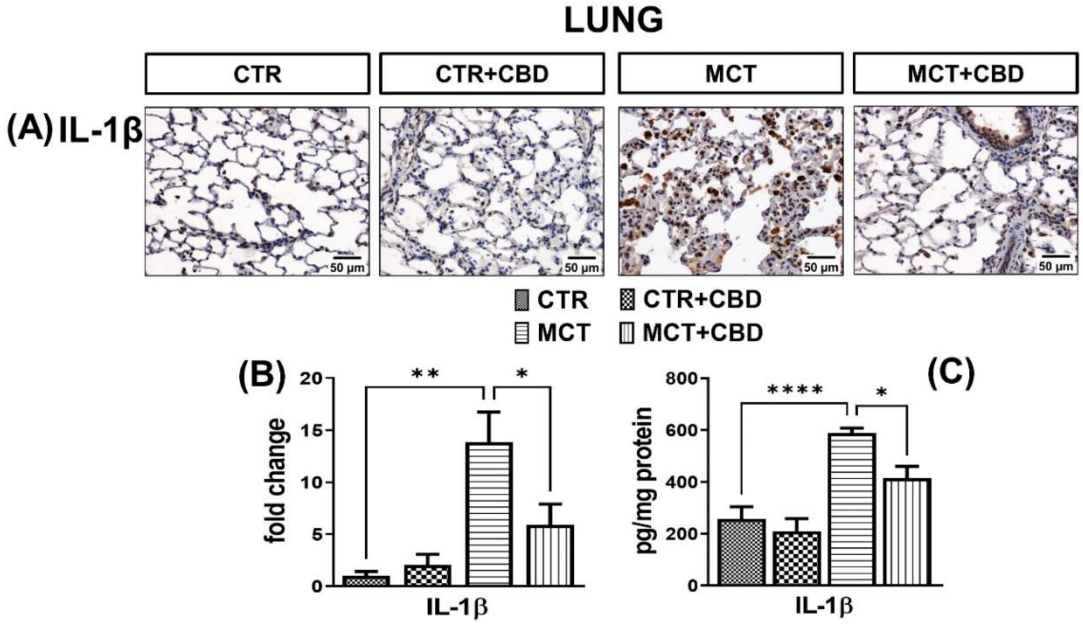

Figure 3. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on interleukin-1β (IL-1β) (**A**); representative micrographs of immunohistochemical staining (magnification 200×) (**B**) and their quantification; (**C**) the concentration of IL-1β determined by enzyme-linked immunosorbent assay (ELISA) in rats' lungs. Bar graphs (**B**,**C**) illustrate the fold changes (for the relative fold change in expression in comparison to the respective CTR, whose expression level was set to 1) in the percentage area stained for IL-1β and concentration of IL-1β, respectively. The dark brown precipitate represents the intensity of IL-1β. CBD (10 mg/kg) or its vehicle was injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 6–7 per group); * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$, compared to the respective group.

Chronic administration of CBD to MCT-treated rats reduced IL-1β immunoreaction intensity by about 57% (Figure 3A,B), and its concentrations by about 30% (Figure 3C). No differences were observed in immunoreactivity or in concentrations of IL-1β in the lungs of CTR animals receiving CBD (Figure 3).

The immunohistochemical reaction using the anti-CD68 antibody showed a greater number of macrophages, with an approximately two-fold greater immunoreactivity in the lungs of MCT rats compared to CTR (Figure 4A,B). The density of CD68 analysed by Western blot method was increased in the lungs of MCT rats by about two-fold (Figure 4C).

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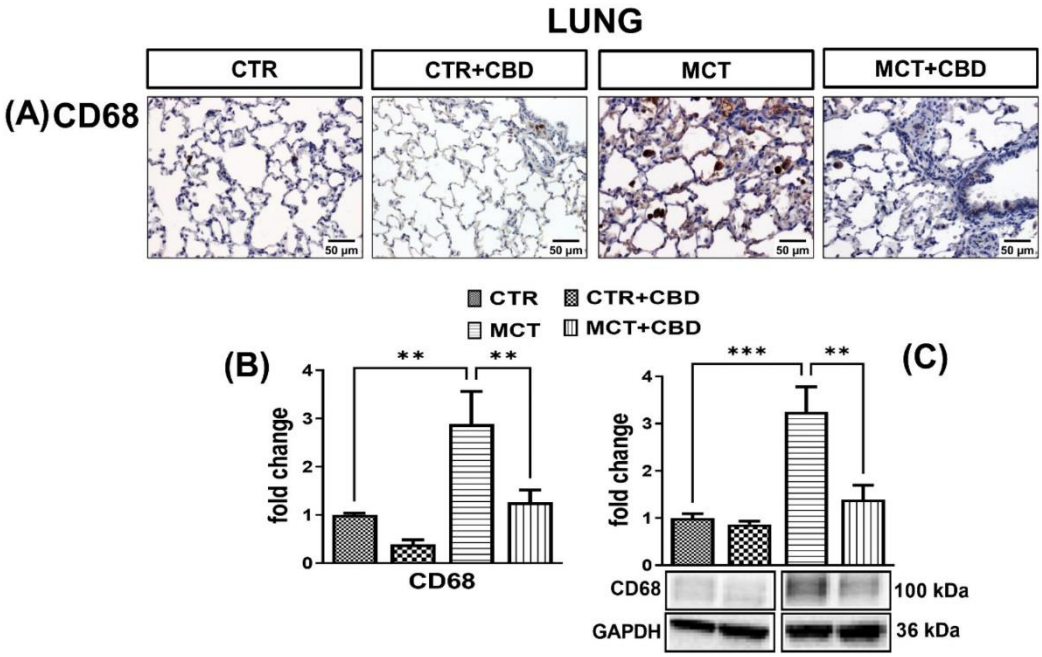

Figure 4. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on cluster of differentiation (CD68) (**A**); representative micrographs of immunohistochemical staining (magnification 200×) (**B**) and their quantification; and (**C**) the density of CD68 determined by Western blot in rats' lungs. Bar graphs B and C illustrate the fold changes (for the relative fold change in expression in comparison to the respective CTR, whose expression level was set to 1) in the percentage area stained for CD68 and in the density of CD68, respectively. The dark brown precipitate represents the intensity of CD68. CBD (10 mg/kg) or its vehicle was injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 6–7 per group); ** $p < 0.01$, *** $p < 0.001$, compared to the respective group.

Administration of CBD to MCT-treated rats decreased the number of CD68-immunopositive cells (Figure 4A), the intensity of the immunohistochemical reaction (Figure 4B), and the density (Figure 4C) of CD68 by about 56% in both cases. In the lungs of CTR and CTR + CBD rats, weak CD68 immunoreactivity was observed only in single cells (Figure 4A).

2.4. Influences of PH and Chronic Administration of CBD on Expression of Cannabinoid Receptors in Lung Tissue

CB_1-R (Figure 5A,B) and CB_2-R (Figure 6A,B) immunostaining in the lungs of MCT rats results in a strong response in the cells. The densities of CB_1-Rs (Figure 5C) and CB_2-Rs (Figure 6C) determined by Western blot method were increased (by about 160% and 100%, respectively) in the lungs of MCT rats.

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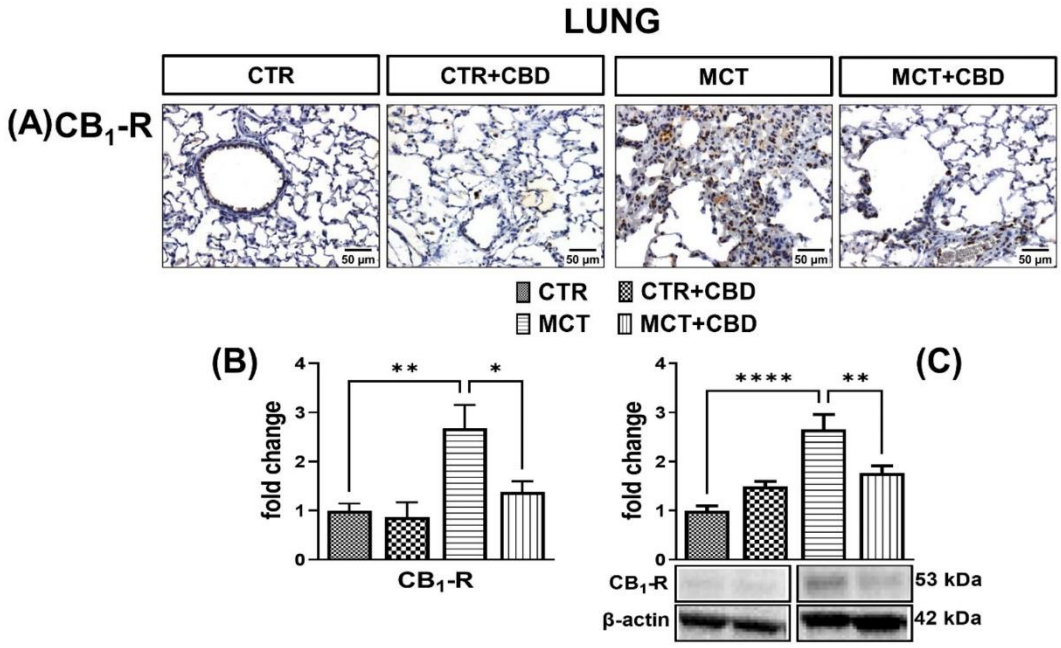

Figure 5. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on cannabinoid receptor type 1 (CB_1-R) (**A**); representative micrographs of immunohistochemical staining (magnification 200×) (**B**) and their quantification; and (**C**) the density of CB_1-R determined by Western blot in rats' lungs. Bar graphs B and C illustrate the fold changes (for the relative fold change in expression in comparison to the respective CTR, whose expression level was set to 1) in the percentage area stained for CB_1-R and in the density of CB_1-Rs, respectively. The dark brown precipitate represents the intensity of CB_1-R. CBD (10 mg/kg) or its vehicle was injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 6–7 per group); * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, compared to the respective group.

Administration of CBD to MCT-treated rats decreased the immunoreactivity (Figure 5A,B) and density (Figure 5C) in CB_1-Rs by about 50% and 34%, respectively, but CB_2-R expression was unchanged in both cases (Figure 6A–C). After administration of CBD to MCT rats, we only observed a downward trend of CB_2-Rs expression (Figure 6B). No changes of these receptors were observed in CTR rats treated with CBD (Figures 5 and 6).

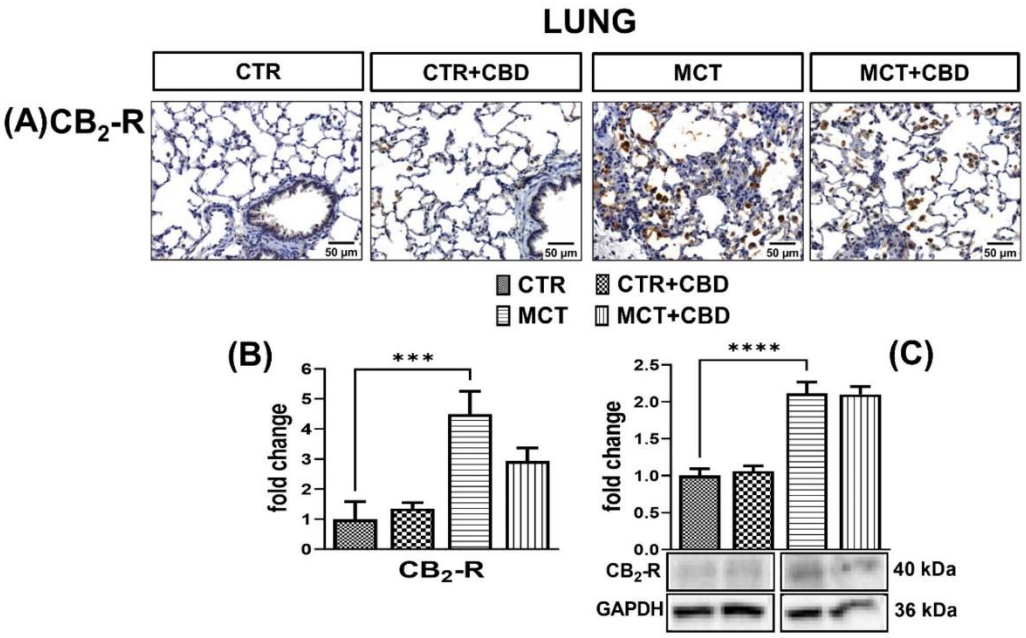

Figure 6. The influence of monocrotaline (MCT)-induced pulmonary hypertension and cannabidiol (CBD) or its vehicle on cannabinoid receptor type 2 (CB_2-R) (**A**); representative micrographs of immunohistochemical staining (magnification 200×) (**B**) and their quantification; and (**C**) the density of CB_2-R determined by Western blot in rats' lungs. Bar graphs B and C illustrate the fold changes (for the relative fold change in expression in comparison to the respective CTR, whose expression level was set to 1) in the percentage area stained for CB_2-R and in the density of CB_2-Rs, respectively. The dark brown precipitate represents the intensity of CB_2-R. CBD (10 mg/kg), or its vehicle was injected *i.p.* every 24 h for 21 days. Data are presented as mean ± SEM, (n = 6–7 per group); *** $p < 0.001$ and **** $p < 0.0001$, compared to the respective group.

3. Discussion

The aim of the study was to evaluate whether chronic administration of CBD had a beneficial effect on the parameters of oxidative stress and inflammation in rats with MCT-induced PH. In addition, we present for the first time how MCT-induced PH affects cannabinoid receptors (CB_1-R and CB_2-R) in rat lung tissues, and how chronic CBD administration regulates the expressions of these receptors.

The experiments were performed on lung tissue from animals with MCT-induced PH. We used this model because it well reflects changes in human's PH, and causes selective damage to pulmonary circulation. The MCT model is an inflammatory model that has been used in experimental studies with drugs already used in the standard treatment of PH [18].

We administered CBD at a dose of 10 mg/kg for 21 days *i.p.* This dose was established based on literature data, where chronic administration of CBD has shown positive effects; it was calculated that this dose corresponds to a dose of 800 mg for a person weighing 80 kg [20]. CBD in the same dose reduced RVSP, pulmonary arterial and right ventricular hypertrophy [15,16]. Chronic administration of CBD, at a dose of 10 mg/kg modified the levels of oxidative stress parameters, changed the expression of CB-Rs in the hearts and plasma [21], and improved vasodilation in aortas and small mesenteric arteries [22] in rats with primary and secondary hypertension. Additionally, in the mouse autoimmune myocarditis model, the administration of CBD at a dose of 10 mg/kg for 46 days *i.p.* improved the contractile function of the heart and furthermore reduced inflammation [23].

3.1. Influence of PH and Chronic CBD Administration on Antioxidant Status

Redox imbalance to the detriment of antioxidant substances is one of the features of PH. Oxidative stress contributes mainly to the dysfunction of the pulmonary endothelium, which results in increased resistance in pulmonary circulation. Excessive production of reactive oxygen species (ROS) contributes to the reduced nitric oxide bioavailability, and thus intensifies the contraction and remodeling of pulmonary vessels [24]. In our study, we observed reduced levels of total antioxidant capacity (TAC) and GSH in the lung tissue of rats with PH. Glutathione is a powerful antioxidant that is involved in regulating the content of reactive nitrogen species (RNS) and ROS. Excess ROS and RNS can damage proteins and lipids [25]. We also observed an increased concentration in 4-HNE in the lungs of rats with PH, which is the main product of lipid peroxidation resulting from increased oxidative stress. The increase in 4-HNE may be related to a decrease in GSH, since under normal conditions 4-HNE is neutralized by glutathione S-transferase which conjugates 4-HNE to GSH [26]; with reduced GSH levels, neutralization of 4-HNE may become less efficient. In the plasma of rats with MCT-induced PH, an increased development in oxidative stress and lipid peroxidation products is observed [27]. Importantly, ROS levels are three times higher in plasma from patients with PH [28], and the mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) are responsible for the production of ROS in cardiovascular disorders including PH [24].

Reduced antioxidant activity is closely related to the pathogenesis of PH, and CBD is a compound with antioxidant potential (see Introduction). In our study, we observed an increase in TAC after chronic administration of CBD. Similarly, GSH concentration and GSR activity increased after CBD administration. The role of GSR is to maintain an appropriate concentration of GSH; therefore, CBD probably increases the activity of the GSR enzyme, also increasing the level in GSH. Our results are consistent with previous reports of a particularly pronounced increase in GSH levels in the myocardial tissues of mice with diabetic cardiomyopathy after CBD administration [29]. Chronic administration of CBD in the same dose (10 mg/kg) increased the levels in GSH in the plasma and heart of rats with primary and secondary hypertension, and increased the level in vitamin E in the plasma of rats with secondary hypertension [21]. Additionally, CBD recovered the dysfunctional mitochondria in hypoxia condition and reduced oxidative stress and excessive glycolysis induced by hypoxia in human PH-PASMC cell culture [16]. CBD is also known to support the action of antioxidant enzymes by preventing the depletion of elements such as zinc and selenium, which are usually lowered in pathological conditions. These elements are essential for the biological activity of antioxidant enzymes. In addition, CBD, by lowering the levels in ROS, prevents the oxidation of GSH and other non-enzymatic antioxidants [7].

3.2. Influence of PH and Chronic CBD Administration on Inflammation

Higher levels of proinflammatory cytokines in lung tissue and/or plasma have been confirmed in human PH and in PH animal models [4]. It has also been suggested that the uncontrolled secretion of inflammatory mediators is one of the factors enhancing lung remodeling in human PH [30]. In our study, there seems to be a relationship between the NF-κB pathway, and the inflammatory condition associated with PH. The increase in NF-κB concentration in the lung tissue of rats with PH is associated with an increase in proinflammatory cytokines, i.e., TNF-α and IL-1β, and an increase in MCP-1 is also noticeable. NF-κB is known to be a central regulator of the genes responsible for the development of inflammation in PH [31]. As mentioned above, ROS promote the development of PH by activation of the NF-κB pathway and the activator protein 1 (AP-1), thus stimulating the growth of MCP-1, which is responsible for the infiltration of monocytes and macrophages. Infiltrating inflammatory cells increases the secretion of inflammatory cytokines TNF-α and IL-1β [32]. Our results are consistent with previous reports in which rats with hypoxia-induced PH showed an increase in NF-κB, IL-1β and TNF-α parameters [33].

We confirmed the increased infiltration of monocytes and macrophages using two independent methods. Activated CD68-labeled macrophages play an important role in

remodeling pulmonary vessels. Macrophages are a source of leukotriene B4 (LTB4), which is responsible for damaging the endothelium of the pulmonary arteries and consequently, increasing the proliferation and hypertrophy of smooth muscle cells. LTB4 blockade is effective in improving pulmonary vascular function, and a reduction in CD68 macrophages reduces the development of SU5416-induced PH in rats [34]. In our immunohistochemistry study we demonstrated increased infiltration of macrophages in the perivascular spaces. We observed that the alveolar macrophages of the MCT rats showed morphological signs of activation, such as enlargement and growth of intracellular vacuoles predominantly in lung interstitial tissue. Thus, we confirmed the results of Xu et al. [35] who, in addition to showing increased expression in CD68 in the lung tissue of rats with MCT-induced PH, showed that macrophage infiltration increased with the development of PH and was the highest on the 28th day of the experiment.

We found no differences in COX-2 expression in the CTR rats and MCT-treated rats. Our results are closest to those reported by Seta et al. [36], who demonstrated no differences in the rats' lung tissue between CTR and MCT-induced PH. Literature data on COX-2 expression are unclear, since cyclooxygenase 1 (COX-1) and COX-2 were strongly overexpressed in human lung tissues [37]; in the lungs of hypoxic-induced PH rats, an increased expression in COX-2 with unchanged COX-1 expression was detected [38]. Supposedly, this may be due to the differences in models used in the experiments, as well as the length of induction of PH (in humans, the development of PH takes many years).

Excessive inflammation mediates further stages of pulmonary vascular remodeling in human PH and animal PH models, as discussed in more detail in Rabinovitch et al. [34]. CBD is a compound with anti-inflammatory properties, but the mechanisms of action are not fully understood [6]. In our preventive model, CBD reduced the concentrations in TNF-α and IL-1β, which are dependent on the NF-κB pathway, as well as MCP-1 and CD68, which are associated with macrophage infiltration. A higher lung TNF-α level determined by mRNA expression was shown in the Sugen-hypoxia mouse model [16], however, as mRNA levels do not always reflect protein levels [39], we confirmed these results based on the protein level in the lungs of rats with MCT-induced PH. Our results confirm that chronic administration of CBD down-regulates inflammation, and further suggest that the anti-inflammatory properties of CBD are related to the NF-κB pathway. Moreover, the NF-κB pathway also participates in the activation of MCP-1 genes. In our research, the decrease in the concentration of NF-κB after chronic administration of CBD was correlated with a decrease in the concentration of MCP-1 in lung tissues. Presumably, inhibition of the NF-κB pathway by CBD is associated with reduced infiltration of monocytes and macrophages into the perivascular tissue of the lungs, limiting the development of inflammation, and therefore the unfavourable remodeling of pulmonary vessels. The above-mentioned notion is also confirmed by our analysis, where CBD decreased CD68 expression which is widely expressed in macrophages. Kozela et al. [40] showed that CBD reduced the levels of proinflammatory IL-1β and IL-6 in LPS-activated BV-2 microglial cells, and that this reduction was not dependent on CB_1-Rs/CB_2-Rs. The authors suggested that CBD can reduce the level of cytokines, mainly IL-1β and IL-6, via the NF-κB-dependent pathway by inhibiting the phosphorylation of the NF-κB p65 subunit [40]. CBD also reduced TNF-α concentration and NF-κB activity in RAW 264.7 macrophages [41]. Muthumalage and Rahman [42] also observed a reduction in MCP-1 in human bronchial epithelial cells after CBD administration, and suggested that this may be related to the reduction in NF-κB activity.

The NF-κB pathway has been suggested to be associated with the regulation of the proinflammatory *COX-2* gene. We did not observe the influence of CBD on the density of COX-2 in the lungs of rats with PH; however, acute inhibition of COX-2 can be dangerous in the early stages of the disease. COX-2 is one of the mediators of the reaction in which compounds with a vasodilating effect on the pulmonary vessels are also formed (prostacyclin). Fredenburgh et al. [43] even suggested that deficiency or pharmacological blocking of COX-2 exacerbates hypoxia-induced PH in mice.

3.3. Influence of PH and Chronic CBD Administration on Expression of Classic Cannabinoid Receptors

In our study, we have shown for the first time the overexpression of CB_1-Rs and CB_2-Rs in PH. Increased CB_2-R density in lung tissue is probably associated with the presence of numerous inflammatory cells known to express CB_2-Rs to a great extent [6,13]. Moreover, it has been suggested that TNFα and IL-1β can directly upregulate CB_1-Rs and CB_2-Rs in human blood [44]. It is also noteworthy that the activation of CB_1-Rs intensifies the proinflammatory response, especially related to TNF-α, and is associated with increased oxidative stress through increased production of ROS. On the contrary, in the case of the CB_2-R, its activation reduces the amount of ROS and TNF-α [7]. The endocannabinoid system seems to play a role in PH, although it is not entirely clear how significant it is. In our previous work, [15] we have shown that *N*-arachidonoyl glycine (NAGLy) and palmitoleoyl ethanolamide (POEA) levels are reduced in the lung tissue of rats with MCT-induced PH, and NAGLy is an endocannabinoid with systemic vasodilating potential [13].

We have shown that CBD reduces the density of CB_1-Rs in the lungs of rats with PH. The proinflammatory effect of CB_1-Rs was recently confirmed by Haddad [45], where administration of arachidonyl-2′-chloroethylamide (ACEA), a selective agonist of CB_1-Rs, caused an increase in IL-6 concentration in rat skeletal muscle myotubes. One of the mechanisms of such regulation may be the Gi/PI3K–Akt/NF-kB pathway. Notably, the activation of CB_1-Rs participates in damage and inflammation of the lungs through IL-1β, TNF-α and MCP-1 signaling [11,46]. Thus, it appears that reduction in CB_1-R expression is protective in PH.

In our study, CBD did not change the CB_2-R density which was increased by PH. However, the role of this receptor in PH is still unclear. Administration of the CB_2-R antagonist (AM630) increased IL-6 expression in mice PASMCs, but no differences in the PH phenotype were noted between the wild type PH mice and *Cnr2*$^{-/-}$ mice with PH [16].

In our study, we did not find that CBD influenced the parameters of oxidative stress and inflammation in healthy (CTR) animals. Therefore, it seems that activity of CBD might be limited to the pathological conditions, while remaining neutral in healthy animals. Overall, CBD is considered a well-tolerated and safe drug with additional pharmacological benefits, including a relaxing effect, administration by inhalation and lack of influence on systemic pressure [6], all of which make it an interesting agent to investigate in PH therapy.

3.4. Limitations of the Study

The present study was limited to the examination of the effects of CBD on parameters of oxidative stress and inflammation in the lung tissue of male rats. As PH is a disease that develops especially in women, it would be appropriate to extend the research to female rats [47]. Additionally, we only used one model of PH in our study. Moreover, in the future, the antioxidant and anti-inflammatory effects of CBD in lung tissue could also be extended to receptor research by administering receptor antagonists, which may modulate the effects of CBD.

4. Materials and Methods

4.1. Animals

All experimental protocols were conducted in accordance with the Local Animal Ethics Committee in Olsztyn (Poland, project code: 88/2018, approved 27 November 2018) and the European Directive (2010/63/EU). The experiments were performed following the principles of replacement, refinement or reduction. Animals were obtained from the Centre of Experimental Medicine of the Medical University of Bialystok (Bialystok, Poland). Animals were kept under a 12 h/12 h light/dark cycle and had free access to food and water. The experiments were performed on 40 male Wistar rats (5–8 weeks old, with body weights of 150–250 g).

4.2. Monocrotaline and CBD Treatment

Wistar rats were injected MCT with a volume of 3 mL/kg at the dose of 60 mg/kg body weight once subcutaneously (*s.c.*) on day "0", or with vehicle for MCT at the same dose. MCT was dissolved in 1 mol/L hydrochloric acid neutralized with 1 mol/L sodium hydroxide and diluted with saline. Healthy rats as well as rats treated with MCT were injected intraperitoneally (*i.p.*) with CBD (10 mg/kg) or its vehicle (ethanol, Tween 80, 0.9% NaCl—3:1:16) every 24 h for 21 days in the following 4 groups: (1) MCT: rats treated with MCT and vehicle for CBD; (2) MCT + CBD: rats treated with MCT and CBD; (3) control (CTR): rats treated with MCT vehicle and CBD vehicle; and (4) CTR + CBD: rats treated with CBD and MCT vehicle. For the sake of simplicity, the group injected with solvents for MCT and CBD was labelled as CTR. Day 21 was selected as the endpoint of the experiments based on our preliminary study that found that rats at day 21 already had well-developed PH, and as was previously described by Chen et al. [48]. The animals were anesthetized with sodium pentobarbital (300 µmol/kg, *i.p.*). A pressure catheter (SPR-320 Mikro-Tip, Millar, Houston, TX, USA) was inserted through the right jugular vein using the closed chest method. Measurements were taken from the right ventricle and recorded on a LabChart 7.3.7 Pro (ADInstruments, Hastings, UK) [14].

4.3. Tissue Preparation for Biochemical and Immunohistochemistry Examinations

Rats were anesthetized with pentobarbital sodium (300 µmol/kg, *i.p.*) 24 h after the last dose of CBD or its vehicle to collect lung tissues. Following thoracotomy, the lungs and the trachea were collected in whole. After the left lung was cut off, the right lung was fixed in 10% buffered formalin by injecting a fixative with a syringe into the right main bronchus until the pleura was smooth, and fixed in formalin for 72 h at +4 °C. After fixation, the same fragments were excised from each lung and embedded in paraffin in the routine manner. Paraffin blocks were cut into 4-micrometer sections and stained with haematoxylin and eosin (H + E) for general histological evaluation. On 4-micrometer paraffin sections, immunohistochemical reactions were performed using specific antibodies. Left lungs were perfused with 0.9% saline and were snap-frozen with liquid nitrogen and stored at −80 °C for biochemical examinations.

4.4. Western Blot

Frozen lungs were weighed, powdered and homogenized in Mammalian Protein Extraction Reagent (MPER, Thermo Scientific, Rockford, IL, USA) that contained a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The total protein concentration was determined using the bicinchoninic acid method (Price Rapid Gold BCA, Protein Assay Kit, Thermo Scientific, Waltham, MA, USA), with bovine serum albumin as a standard. Next, homogenates were reconstituted in Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and blocked in EveryBlot Blocking Buffer (Bio-Rad Laboratories, Inc., Tokyo, Japan). The membranes were incubated overnight at 4°C with corresponding primary antibodies in appropriate dilutions (i.e., CB1-R (1:500; Abcam, Cambridge, UK), CB2-R (1:500; Abcam, Cambridge, UK), CD68 (1:500; Abcam, Cambridge, UK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000; Abcam, Cambridge, UK), cyclooxygenase 2 (COX-2) (1:500; Abcam, Cambridge, UK) and β-actin (1:3000; Abcam, Cambridge, UK)). In order to detect proteins, anti-rabbit primary and anti-goat IgG horseradish peroxidase-conjugate secondary antibodies (1:3000; Abcam, Cambridge, UK) were used. After adding a suitable substrate for horseradish peroxidase (Clarity Western ECL Substrate; Bio-Rad Laboratories, Inc., Santa Cruz, CA, USA), the protein bands were quantified densitometrically using a ChemiDoc visualization system (Image Laboratory Software Version 6.0.1; Bio-Rad, Warsaw, Poland). The levels of the protein detected were normalized to β-actin or GAPDH.

4.5. ELISA/Colorimetric Assays

The concentrations of MCP-1, IL-1β, TNF-α and NF-κB (p65) were measured using an enzyme-linked immunosorbent assay (ELISA). Total antioxidant capacity (TAC) was determined with the ImAnOx Kit from Immundiagnostik (Bensheim, Germany) according to the manufacturer's protocol. The reaction is based on the elimination of exogenous hydrogen peroxide by antioxidants contained in the sample. The difference between the hydrogen peroxide added and that remaining after the reaction is proportional to the TAC. Hydrogen peroxide was measured at 450 nm absorbance. Quantification was done with the provided calibrator. All measured parameters were normalized for the concentration of total protein using the bicinchoninic acid method (Price Rapid Gold BCA, Protein Assay Kit, Thermo Scientific, Waltham, MA, USA), with bovine serum albumin as a standard.

4.6. Immunohistochemistry

Immunostaining was performed by the following protocol: paraffin-embedded sections were deparaffined and hydrated in pure alcohols. For antigen retrieval, the sections were subjected to pretreatment in a pressure chamber and heated using Target Retrieval Solution Citrate pH = 6.0 (Agilent Technologies, Inc. Santa Clara, CA, USA). After cooling down to room temperature, the sections were incubated with Dako REAL Peroxidase-Blocking Solution (Agilent Technologies, Inc. Santa Clara, CA, USA). The sections with the primary antibodies IL-1β (1:1000; Abcam, Cambridge, UK), CD68 (1:1000; Agilent Technologies, Inc. Santa Clara, CA, USA), CB1-R (1:1000; Abcam, Cambridge, UK) and CB2-R (1:200; Abcam, Cambridge, UK), were incubated 24 h at +4 °C in a humidified chamber. This procedure was followed by incubation with secondary antibody (EnVision FLEX, High pH (Link), HRP. Rabbit/Mouse, Agilent Technologies, Inc., Santa Clara, CA, USA). The bound antibodies were visualized by incubation with DAB Flex chromogen. The sections were finally counterstained in hematoxylin QS (Vector Laboratories, Burlingame, CA, USA), mounted and evaluated under light microscope. Sections were dehydrated, and the specificity of the antibodies was confirmed using a negative control, where the antibodies were replaced by Antibody Diluent (Vector Laboratories, Burlingame, CA, USA). The results of staining were submitted for evaluation in an Olympus BX43 microscope with Olympus DP12 camera. In each lung sample, the percentage area stained was measured using the ImageJ software version 1.53c (NIH, Bethesda, MD, USA) [49], and an average was calculated from the values obtained from the six lungs per group. In order to calculate the percentage area of staining in the lungs, the area stained was divided by the total area of lung tissue to obtain a percentage staining. The data were presented as the fold changes compared to the respective CTR group.

4.7. Determination of Antioxidant Enzyme Activity

The method of Mize and Langdon (previously described in [21]) was used to determine the glutathione reductase (GSR—EC.1.6.4.2) activity in lung tissue. One unit of enzyme oxidized 1 µmol of nicotinamide adenine dinucleotide phosphate (NADPH) for 1 min at 25 °C and pH 7.4. Specific enzyme activity was expressed in units per mg of protein.

Glutathione peroxidase (GPx—EC.1.11.1.9) activity was measured spectrophotometrically basing on the method of Paglia and Valentine (previously described in [21]). One unit of GPx activity was determined as the amount of enzyme catalyzing the oxidation of 1 µmol NADPH to NADP+ for 1 min at 25 °C and pH 7.4. Specific enzyme activity was expressed in units per mg of protein.

4.8. Determination of Non-Enzymatic Antioxidant Level

The capillary electrophoresis (CE) method (as previously described in [21]) was used to determine reduced glutathione (GSH) level. Samples were sonicated with 2 mL of a solution of ACN/H_2O (62.5:37.5, v/v), and centrifuged at 29,620× g for 10 min. The separation was performed on a capillary with a total length of 50 cm (40 cm effective

length) and an inner diameter of 50 µm, and was operated at 27 kV with UV detection at 200 ± 10 nm. The concentration of GSH was expressed as nmol per mg tissue.

4.9. Determination of Lipid Modifications

4-HNE was determined by Gas Chromatography-Tandem Mass Spectrometry (GC/MS/MS), as the O-PFBoxime-TMS derivatives (previously described in [21]). Benzaldehyde-D6 was added to the lung lysates, and aldehydes were derivatized by the addition of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxyamine hydrochloride. Samples were deproteinized by the addition of 1 mL of methanol, and OPFB-oxime aldehyde derivatives were extracted by the addition of 2 mL of hexane. The top hexane layer was transferred into borosilicate tubes and evaporated under a stream of argon gas, followed by the addition of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide in 1% trimethylchlorosilane. Derivatized aldehydes were analyzed using a 7890A GC—7000 quadrupole MS/MS (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-5 ms capillary column. Derivatized aldehydes were detected in the selected ion monitoring mode. The ions used for 4-HNE-PFB-TMS identification were m/z 333.0 and 181.0. The level of 4-HNE was expressed in nmol per mg tissue.

4.10. Statistical Analysis

All results are expressed as the mean ± SEM of n animals. GraphPad Prism version 9.3.0 (GraphPad Software, San Diego, CA, USA) was used to plot the mean data. Prior to statistical analysis, all data were analysed for Gaussian distribution, then parametric tests based on validated normality tests were performed. Statistical comparisons between groups were performed using analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests for all data sets. Post hoc tests were performed only when F reached the required level of statistical significance, and no significant homogeneity of variance was found. Differences were considered statistically significant if $p < 0.05$.

4.11. Drugs

(−)-cannabidiol (CBD) (THC-1073G-1) from THC Pharm, Frankfurt, Germany; ethanol (BA6420113) and natrium chloride (NaCl) (BA4121116) from POCH, Gliwice, Poland; Tween 80 (P1754), Crotaline (MCT; C2401-1G) and Tween 80 (P1754) from Sigma-Aldrich, Munich, Germany; MCT vehicle-1 M HCl, and the pH was adjusted to 7.4 with 1 M NaOH; pentobarbital sodium (5909991290153) from Biowet, Pulawy, Poland; Mammalian Protein Extraction Reagent (M-PER; 78501) from Thermo Scientific, Rockford, IL, USA; protease inhibitors (11836153001) from Roche Diagnostics GmbH, Mannheim, Germany; Price Rapid Gold BCA, Protein Assay Kit (A53225) from Thermo Scientific, Waltham, MA, USA; Laemmli buffer (1610737) from Bio-Rad Laboratories, Inc., Hercules, CA, USA; EveryBlot Blocking Buffer (12010020) from Bio-Rad Laboratories, Inc., Tokyo, Japan; Clarity Western ECL Substrate (102031593) from Bio-Rad Laboratories, Inc., Santa Cruz, CA, USA; CB1 (ab259323) from Abcam, Cambridge, UK; CB2 (ab3561) from Abcam, Cambridge, UK; CD68 (ab125212) from Abcam, Cambridge, UK; GAPDH (EPR16891) from Abcam, Cambridge, UK; β-actin (ab8227) from Abcam, Cambridge, UK; Goat Anti-Rabbit IgG H&L (ab6721) from Abcam, Cambridge, UK; ImAnOx (TAS/TAC) Antioxidative Capacity (KC5200) from Immundiagnostik, Bensheim, Germany; IL-1β (670.040.096) ELISA Kit from Immundiagnostik, Bensheim, Germany; TNF-α (865.000.096) ELISA Kit from Immundiagnostik, Bensheim, Germany; Nuclear Factor Kappa B ELISA Kit (SEB824Ra) from Immundiagnostik, Bensheim, Germany; Dako REAL Peroxidase-Blocking Solution (S2023) from Agilent Technologies, Inc., Santa Clara, CA, USA; IL-1β (ab9722) from Abcam, Cambridge, UK; CD68 (M0876) from Agilent Technologies, Inc., Santa Clara, CA, USA; EnVision FLEX, High pH (Link), HRP. Rabbit/Mouse (K800021-2) from Agilent Technologies, Inc., Santa Clara, CA, USA; hematoxylin QS (H-3404) from Vector Laboratories, Burlingame, CA, USA; Wash Buffer (S3006) from Agilent Technologies, Inc., Santa Clara, CA, USA; CB1-R (ab23703) from Abcam, Cambridge, UK; CB2-R (ab3561) from Abcam, Cambridge, UK.

5. Conclusions

The evidence presented the antioxidant and anti-inflammatory effects of CBD in the lung tissue of rats with MCT-induced PH; as well, we reported on its relaxing effect on pulmonary vessels and the properties of reducing RVSP, suggesting that CBD could be a successful adjunctive therapy in the treatment of PH. The NF-κB pathway and downregulation of CB_1-Rs, the activation of which has pro-oxidative and proinflammatory effects, may play a special role in the antioxidant and anti-inflammatory CBD-mediated effect. The available therapies for PH treatment are focused only on vascular effects; CBD has multipotent beneficial effects, which is in line with the current trend of seeking multidirectional therapies. The promising results of our research may form the basis for a more detailed study of the effects of CBD or its derivatives on PH, especially in humans.

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Sample Availability: Samples of the compounds are not available from the authors.

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Rozdział 12. Druga praca oryginalna

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Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats

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ABSTRACT

Cannabidiol (CBD) is a non-intoxicating compound of Cannabis with anti-fibrotic properties. Pulmonary hypertension (PH) is a disease that can lead to right ventricular (RV) failure and premature death. There is evidence that CBD reduces monocrotaline (MCT)-induced PH, including reducing right ventricular systolic pressure (RVSP), vasorelaxant effect on pulmonary arteries, and decreasing expression of profibrotic markers in the lungs. The aim of our study was to investigate the effect of chronic administration of CBD (10 mg/kg daily for 21 days) on profibrotic parameters in the RVs of MCT-induced PH rats. In MCT-induced PH, we found an increase in profibrotic parameters and parameters related to RV dysfunction, i.e. plasma pro-B-type natriuretic peptide (NT-proBNP), cardiomyocyte width, interstitial and perivascular fibrosis area, amount of fibroblasts and fibronectin, as well as overexpression of the transforming growth of factor β1 (TGF-β1), galectin-3 (Gal-3), suppressor of mothers against decapentaplegic 2 (SMAD2), phosphorylated SMAD2 (pSMAD2) and alpha-smooth muscle actin (α-SMA). In contrast, vascular endothelial cadherin (VE-cadherin) levels were decreased in the RVs of MCT-induced PH rats. Administration of CBD reduced the amount of plasma NT-proBNP, the width of cardiomyocytes, the amount of fibrosis area, fibronectin and fibroblast expression, as well as decreased the expression of TGF-β1, Gal-3, SMAD2, pSMAD2, and increased the level of VE-cadherin. Overall, CBD has been found to have the anti-fibrotic potential in MCT-induced PH. As such, CBD may act as an adjuvant therapy for PH, however, further detailed investigations are recommended to confirm our promising results.

1. Introduction

Pulmonary hypertension (PH) is a rapidly progressive and still incurable disease and it is diagnosed when the mean pulmonary artery pressure is >20 mmHg [1]. The most important pathogenetic factors of PH include pulmonary endothelial dysfunction, inflammation, oxidative stress, increased resistance of small pulmonary arteries and their remodeling. However, the most serious consequence of pulmonary vascular changes is excessive right ventricular (RV) afterload. Developing heart failure most often leads to worsening prognosis and premature patients' death [2]. Prolonged excessive afterload with the accompanying inflammation and oxidative stress contribute to the pathological remodeling and cardiac fibrosis [3]. Many signaling pathways are activated during this process, including transforming growth factor β1/suppressor of mothers against decapentaplegic (TGF-β1/SMAD) cascade. There is also an increased activation of fibroblasts,

Abbreviations: α-SMA, alpha-smooth muscle actin; ANOVA, one-way analysis of variance; $CB_{1,2}$-R, cannabinoid receptor type 1 and 2; CBD, cannabidiol; CTR, control; DOCA-salt, deoxycorticosterone acetate salt hypertensive rats; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EndoMT, endothelial to mesenchymal transition; FDA, Food and Drug Administration; FGF-2, fibroblast growth factor 2; Gal-3, galectin 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.p., intraperitoneal; MCT, monocrotaline; MMP-9, matrix metalloproteinase-9; NT-proBNP, plasma pro-B-type natriuretic peptide; PH, pulmonary hypertension; pSMAD2, phosphorylated form of suppressor of mothers against decapentaplegic; RV, right ventricle; RVSP, right ventricular systolic pressure; s.c., subcutaneously; SHR, spontaneously hypertensive rats; SMAD 2, suppressor of mothers against decapentaplegic 2; TGF-β1, transforming growth factor beta 1; VE-cahderin, vascular endothelial cadherin.

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which transform into myofibroblasts and produce extracellular matrix (ECM) elements [4,5]. Moreover, it is characteristic to increase the expression of cardiac remodeling and/or dysfunction markers such as plasma pro-B-type natriuretic peptide (NT-proBNP) [6] or alpha-smooth muscle actin (α-SMA) and galectin-3 (Gal-3) in the RV [5,7]. Extensive ventricular fibrotic remodeling abolishes the contractility of the heart and is associated with the loss of a large number of cardiomyocytes, eventually replacing the dead heart muscle with a collagen scar. The consequence of the above-mentioned processes is the deterioration of the systolic-diastolic function of the RV and a number of consequences of the development of heart failure [3].

Currently available drugs do not cure the disease and are mainly aimed at pulmonary vasodilation [8]. Given that RV failure in the PH is one of the factors contributing to the high mortality rate, it is undoubtedly necessary to focus on this aspect of the disease. Targeting new regulators of cardiac fibrosis is considered an effective strategy for the clinical treatment of PH.

Cannabidiol (CBD) is a non-intoxicating compound isolated from *Cannabis sativa* var. *indica*, that is well tolerated and safe, and has been approved by the Food and Drug Administration (FDA) for the treatment of drug-resistant epilepsy [9]. CBD has been shown to have several beneficial properties in limiting changes in PH [9]. These effects include: reduction of right ventricular systolic pressure (RVSP), reduction of pulmonary arterial hypertrophy in the rat monocrotaline (MCT)-induced PH model and/or in the mouse Sugen-hypoxia-induced PH model [10,11]. Moreover, CBD reduces inflammation and improves the antioxidant capacity in lungs of MCT-induced PH rats [12], and reduces RV hypertrophy and oxidative stress in MCT-induced PH in rats and/or Sugen-hypoxia-induced PH in mouse [11]. In addition, CBD brings benefits to the vascular function, by causing vasorelaxant effect in animal and human pulmonary arteries [13] and improving the pulmonary vascular endothelium function in the MCT-induced PH [10]. Additionally, CBD improved cardiac performance in hearts of spontaneously hypertensive (SHR) and deoxycorticosterone acetate (DOCA)-salt hypertensive rats [14], but neither acute nor chronic intraperitoneal (i.p.) administration of CBD at a dose of 10 mg/kg modified cardiovascular parameters in both models [15,16]. Nevertheless, CBD was effective in reducing oxidative and nitrative stress, and inflammation in the rat [17] and the mouse [18] model of doxorubicin-induced cardiomyopathy, as well as reducing cardiac dysfunction and myocardial fibrosis in the experimental autoimmune myocarditis mouse model [19].

Given the reports of CBD's beneficial effects in alleviating PH in experimental models [10–12], its potential antiproliferative properties, as well as the lack of an effective drug available to improve cardiac function in the severe PH, the aim of the study was to investigate if CBD possesses the antifibrotic potential mechanism in the RV of rats with MCT-induced PH.

2. Material and methods

Experiments were accepted by the Local Ethical Committee for Animal Experiments, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland (project code: 88/2018, approved on November 27, 2018) and were performed in agreement with the European Directive (2010/63/EU) and the ARRIVE guidelines. We decided to use previously preserved tissues for research purposes based on the 3R principle (Refinement, Reduction and Replacement). This paper continues the results of the work presented by Sadowska et al. [10] (Table 1).

Table 1
As described previously [10], the RVSP in groups were:

CTR	20.03 ± 0.9 mmHg, $n = 10$,
CTR + CBD	21.4 ± 0.9 mmHg, $n = 10$
MCT	43.7 ± 3.9 mmHg, n = 10, $p < 0.001$
MCT + CBD	28.2 ± 0.7 mmHg, n = 10, $p < 0.001$

CBD- cannabidiol; CTR- control group; MCT-monocrotaline.

2.1. Animals

Forty male Wistar rats (5–8 weeks old, 150–250 g) were used in the experiments. Rats were obtained from the Experimental Medicine Centre of the Medical University of Bialystok, Bialystok, Poland. During the 12 h/12 h light/dark cycle, the animals were kept in plastic cages with free access to food and water.

2.2. MCT and CBD treatment

The animals were randomly divided into 4 groups. Two groups of animals were injected with plant-derived alkaloid-monocrotaline (from Sigma-Aldrich, Munich, Germany) at the dose of 60 mg/kg of body weight, once, subcutaneously (s.c.) on the day,0″ in a volume of 3 ml/kg (rats with MCT-inducted PH). And the last two groups of animals were injected with vehicle for MCT (0.9 % NaCl) (rats without PH which were the control group). From day,1″ to day,21″ the animals with and without PH were administered with CBD ((−)-cannabidiol (CBD; THC-1073G-1) THC Pharm, Frankfurt, Germany) at a dose of 10 mg/kg or its vehicle (ethanol, Tween 80, 0.9 % NaCl—3:1:16), i.p., in a volume of 1 ml/kg. Fig. 1 presents the scheme of the experiment.

2.3. Tissue preparation for biochemical and immunohistochemistry examinations

Rats were sacrificed with sodium pentobarbital 1 day after the last dose of CBD or its solvent (300 μmol/kg, i.p.). The whole hearts were collected. The one part of each RV were fixed in 10 % buffered formalin for 72 h at +4 °C and subjected to standard paraffin-embedding procedure. The paraffin blocks were cut into 4 μm sections and stained by hematoxylin and eosin (H + E), by Masson's trichrome and processed by immunohistochemistry for fibronectin (1:2000) and fibroblast growth factor 2 (FGF-2) detection. The remaining parts of RVs were immediately frozen with liquid nitrogen and stored at −80 °C for biochemical studies.

2.4. Histological examinations

Interstitial fibrosis of the right ventricle was determined by quantitative morphometry as previously described [20]. The entire area of all histopathological sections was scanned using a light microscope (Olympus 114 Corp., Tokyo, Japan) with an Olympus DP12 digital camera (Olympus 114 Corp., Tokyo, Japan). About 40 images were selected from each right ventricle. The collagen fraction (stained with Masson's trichrome according to the manufacturer's protocol) was calculated as follows: the total area of interstitial fibrosis was divided by the total area of connective tissue and myocardium of the cross-sectional field of view. We excluded the areas of perivascular fibrosis from this measurement. Perivascular fibrosis was evaluated as the ratio of the blue-stained area surrounding the vessel wall to the total vessel area. The results are presented as the relative fold change in staining in comparison to the respective CTR, which stained area was set to 1.

2.5. Cardiomyocytes width

Histological staining was evaluated in an Olympus BX41 light microscope (Olympus 114 Corp., Tokyo, Japan) with an Olympus DP12 digital camera (Olympus 114 Corp., Tokyo, Japan). From each animal 3 sections of the RV tissue were studied. Five randomly selected microscopic fields from each section (each field of 0.785 mm2; magnification by 200×, 20× by the lens and 10× by the eyepiece) were documented. Subsequently, images were morphometrically evaluated by using the NIS-Elements Advanced Research software of Nikon (Tokyo, Japan). The width of randomly selected 25 cardiomyocytes was estimated for

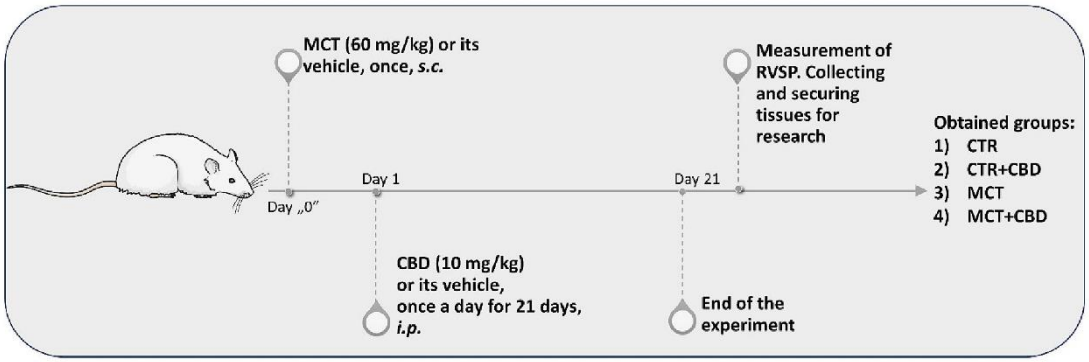

Fig. 1. The scheme of the experiments. CBD- cannabidiol; CTR- control group; i.p.- intraperitoneal injection; MCT-monocrotaline; RVSP- right ventricular systolic pressure; s.c.- subcutaneously injection.

the width in the fields where the long axis in the cleavage plane contained the visible cell nucleus.

2.6. Immunohistochemistry

Immunostaining was performed by deparaffinizing and hydrating paraffin-embedded sections in pure alcohols. The sections of cardiac tissue were subjected to pretreatment in a pressure chamber and heated using Target Retrieval Solution Citrate pH = 9.0 (Agilent Technologies, Inc. Santa Clara, CA, USA). Following cooling to room temperature, the sections were incubated with Dako REAL Peroxidase-Blocking Solution (Agilent Technologies, Inc. Santa Clara, CA, USA). The sections with the primary antibodies: fibronectin (1:2000) and FGF-2 (1:500) were incubated 24 h at +4 °C in a humidified chamber. The next step was incubation with secondary antibody (REAL™ EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse detection kit (Agilent Technologies, Denmark). Visualization of the antibodies was performed by incubation with DAB Flex chromogen. Finally the cardiac sections were counterstained in hematoxylin QS (Vector Laboratories, Burlingame, CA, USA). The specificity of the antibodies was confirmed using a negative control (by replacing the antibodies with an antibody diluent (Agilent Technologies, Denmark)). To evaluate results we used an Olympus BX43 microscope with an Olympus DP12 camera. In each RV sample the percentage area stained was calculated using the ImageJ software version 1.53c (NIH, Bethesda, MD, USA) according to Krzyżewska et al. [12].

2.7. Western blot

The Western blot method was performed according with our previous paper Krzyżewska et al. [12]. Briefly, samples of RV were homogenized in an assay buffer containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, Germany). After SDS polyacrylamide gel electrophoresis and transfer, the membranes were incubated overnight at 4 °C with primary antibodies i.e. Gal-3 (1:5000), matrix metalloproteinase-9 (MMP-9) (1:1000), TGF-β1 (1:1000), pSMAD2 (1:1000), SMAD2 (1:2000), vascular endothelial cadherin (VE-cadherin) (1:200), α-SMA (1:18000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:20000). In the next step, anti-rabbit primary, and anti-goat IgG horseradish peroxidase-conjugate secondary antibodies (1:3000) were used. Substrate (Clarity Western ECL Substrate; Bio-Rad Laboratories, Inc., USA) was added to visualize the bands, and then the bands were assessed by densitometry using the ChemiDoc apparatus (Image Laboratory Software Version 6.0.1; Bio-Rad, Warsaw, Poland). Protein density levels were standardized to GAPDH.

2.8. ELISA

The concentration of NT-proBNP was measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (ELISA kit; MyBioSource, Inc., USA, MBS2881463).

2.9. Statistical analysis

For statistical comparison the GraphPad Prism version 9.3.0 (GraphPad Software, San Diego, California USA) was used. Data were calculated for Gaussian distribution prior to statistical analysis. If the data were normally distributed, the one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test for multiple groups was carried out. Data were subjected to Bonferroni's post hoc tests only when the F value achieved $p < 0.05$ and there was no significant inhomogeneity of variances. The results are shown as the mean ± SEM of n animals. A significance level of $p < 0.05$ was assumed.

2.10. Drugs

(−)-cannabidiol (THC-1073G-1) from THC Pharm, Frankfurt, Germany; GAPDH (EPR16891) from Abcam, Cambridge, UK; Crotaline (MCT; C2401-1G) from Sigma-Aldrich, Munich, Germany; Goat Anti-Rabbit IgG H&L (ab6721) from Abcam, Cambridge, UK; Gal-3 (ab76245) from Abcam, Cambridge, UK; α-SMA (ab124964) from Abcam, Cambridge, UK; VE-cadherin (ab231227) from Abcam, Cambridge, UK; REAL™ EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse detection kit (K5007) from Agilent Technologies, Denmark; SMAD2 (ab40855) from Abcam, Cambridge, UK; pSMAD2 (ab188334) from Abcam, Cambridge, UK; MMP-9 (ab76003) from Abcam, Cambridge, UK; Trichrome Stain (Masson) Kit, (ab150686) from Abcam, Cambridge, UK UK; FGF-2 Polyclonal Antibody (bs-0217R) from Bioss Antibodies Inc., Massachusetts, USA; Anti-TGF-β1 (ab179695) from Abcam, Cambridge, UK; Recombinant Anti-Fibronectin antibody (ab268020) from Abcam, Cambridge, UK; Antibody Diluent with Background Reducing Components (S3022) from Dako, Denmark; hematoxylin QS (H-3404) from Vector Laboratories, Burlingame, CA, USA; NT-proBNP ELISA kit (MBS2881463) from MyBioSource, Inc.San Diego, CA, USA.

3. Results

3.1. Impact of PH and CBD administration on NT-proBNP

The concentration of NT-proBNP was analysed by ELISA method. MCT administration increased the concentration of NT-proBNP (by

about 35 %) in the plasma compared to CTR rats. Chronic administration of CBD to MCT-treated rats decreased the concentration of NT-proBNP (by about 20 %). No changes were observed in CTR rats chronically administered with CBD (Fig. 2).

3.2. Impact of PH and CBD administration on profibrotic parameters

In comparison to CTR rats, the width of cardiomyocytes isolated from MCT rats was increased (by about 75 %) in RVs (Fig. 3A, B). Chronic CBD administration reduced the width of RV myocytes in MCT group (by about 10 %). The Masson's trichrome staining showed an increased right ventricular interstitial area of fibrosis in MCT group (by about 5-fold) and an increased right ventricular perivascular area of fibrosis (by about 2-fold) compared to CTR rats. The chronic CBD administration decreased the right ventricular interstitial area of fibrosis in MCT rats (by about 70 %) and right ventricular perivascular area of fibrosis (by about 60 %) (Fig. 3C-F). No differences in cardiomyocytes width and right ventricular interstitial and perivascular area of fibrosis were observed in CTR rats administered with CBD (Fig. 3B, D, F).

The increased expression of fibronectin and fibroblasts was observed in the RVs of rats treated with MCT by about 10-fold and 2-fold, respectively, compared to CTR rats. In comparison to MCT group the CBD administration to MCT rats decreased the fibronectin immunoreaction and fibroblast expression by about 77 % and 55 %, respectively (Fig. 4A-D).

3.3. Impact of PH and CBD administration on TGF-β1/SMAD2 pathway

The TGF-β1, Gal-3, pSMAD2, SMAD2, α-SMA, VE-cadherin and MMP-9 density in RV was analysed by Western blot method. In the RVs of the MCT group, the expression of TGF-β1, Gal-3, pSMAD2/SMAD2 and α-SMA (Fig. 5A-D) was increased by nearly 120 %, 800 %, 160 % and 40 %, respectively, however, it was decreased for VE-cadherin by nearly 40 % (Fig. 5E), with no differences in MMP-9 between the MCT and CTR groups (Fig. 5F). Chronic CBD administration to MCT-treated rats resulted in a decrease in the density of TGF-β1, Gal-3 and pSMAD2/SMAD2 by about 30 %, 60 % and 40 %, respectively (Fig. 5A, B, C), increase for VE-cadherin by about 40 % (Fig. 5E), and only a tendency toward a reduction for α-SMA (Fig. 5D) and MMP-9 (Fig. 5F). No changes were observed in CTR rats chronically administrated with CBD (Fig. 5A-F).

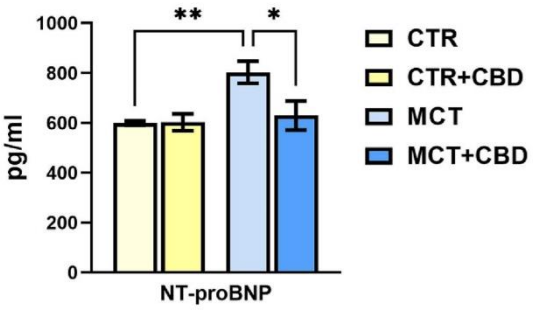

Fig. 2. Cannabidiol (CBD) administration reduced pro-B-type natriuretic peptide (NT-proBNP) in plasma from control (CTR) and monocrotaline (MCT)-induced pulmonary hypertension rats. All data are shown as mean ± SEM, n = 6–7; * $p < 0.05$, ** $p < 0.01$.

4. Discussion

4.1. General

The aim of the study was to investigate if CBD possesses the anti-fibrotic potential in the RV of rats with MCT-induced PH. The studies were performed on RVs that had been obtained from MCT-induced PH rats. We chose this model because it well reflects the changes in human PH. The MCT causes permanent and selective damage to the pulmonary vessels while remaining neutral to the systemic circulation [21]. Importantly, the MCT-induced PH model is one in which RV failure and fibrosis develops fairly well [22].

CBD was administered at a dose of 10 mg/kg of body weight. Such dosage was determined based on literature data showing that chronic administration of CBD reduced RVSP, limited pulmonary vascular hypertrophy, decreased the Fulton index [10,11], and limited inflammation and oxidative stress in lung tissue [12] in the PH model. In addition, CBD at the same dose reduced the width of the cardiomyocytes in the right and left ventricles in SHR and/or DOCA-salt rats [14] as well as improved the contractile function of the heart in the mouse autoimmune myocarditis model [23]. Likewise, chronic administration of CBD at a dose of 10 mg/kg increased the level of glutathione and decreased the level of selected parameters of lipid peroxidation in hearts of SHR and DOCA-salt [16].

4.2. Impact of monocrotaline-induced PH on right ventricle

NT-proBNP is considered to be one of the most important indicators useful in the diagnosis of RV failure in the PH. In addition, it is one of the components of the REVEAL 2.0 risk calculator that is used by clinicians to predict clinical worsening and mortality in patients [6]. In our study, we noticed a higher level of NT-proBNP in the plasma of MCT-induced PH rats. Accordingly, higher levels of NT-proBNP in plasma were also observed in rabbit model of congenital diaphragmatic hernia with developed PH [24] and in rats with MCT-induced PH [25].

Persistent overload with concomitant inflammation leads to pathological remodeling and fibrosis of the heart in PH. The process of remodeling of the RV consists of many interconnected mechanisms, in which especially fibroblasts and the TGF-β1/SMAD2 pathway are involved. A detailed summary of the process of RV fibrosis is presented in Fig. 6 and was described in the Introduction section.

In our experimental model of MCT-induced PH, we showed changes in the RV confirming its developed dysfunction. We observed, among others, increased: width of cardiomyocytes, the area of interstitial and perivascular fibrosis, expression of fibroblasts and fibronectin, as well as density of TGF-β1, Gal-3, pSMAD2, SMAD2 and α-SMA parameters in RV of rats with MCT-induced PH. In contrast, VE-cadherin expression was lower in the RV of above-mentioned group. Fibroblasts are the major effector cells in the process of cardiac fibrosis. Increased expression of α-SMA is a hallmark of mature myofibroblasts [30], which we also demonstrated in the RV of rats with MCT-induced PH, thus confirming in our model the results of Zungu-Edmondson et al. [31] where increased expression of α-SMA was also associated with RV fibrosis in the Sugen-hypoxia-induced PH model in rats. Myofibroblasts secrete large amounts of ECMs, which is confirmed by the increased interstitial and perivascular fibrosis area and increased fibronectin immunoexpression in the RV of MCT-induced PH rats. In this way, we confirm that in the MCT-induced PH model there is increased fibroblast proliferation and pathologically high collagen synthesis in the RV [32]. Similar to our work, the increased percentage of fibrosis area in the RV was also documented using a different staining method (Sirius red) in rat model of the MCT-induced PH and in the Sugen-hypoxia-induced PH [33]. Our analysis showed that the expressions of TGF-β1, SMAD2 and pSMAD2 are higher in the RV of MCT-induced PH rats, confirming the involvement of those components of these pathways in the pathological process of RV remodeling and fibrosis. In the right ventricles of rats subjected to

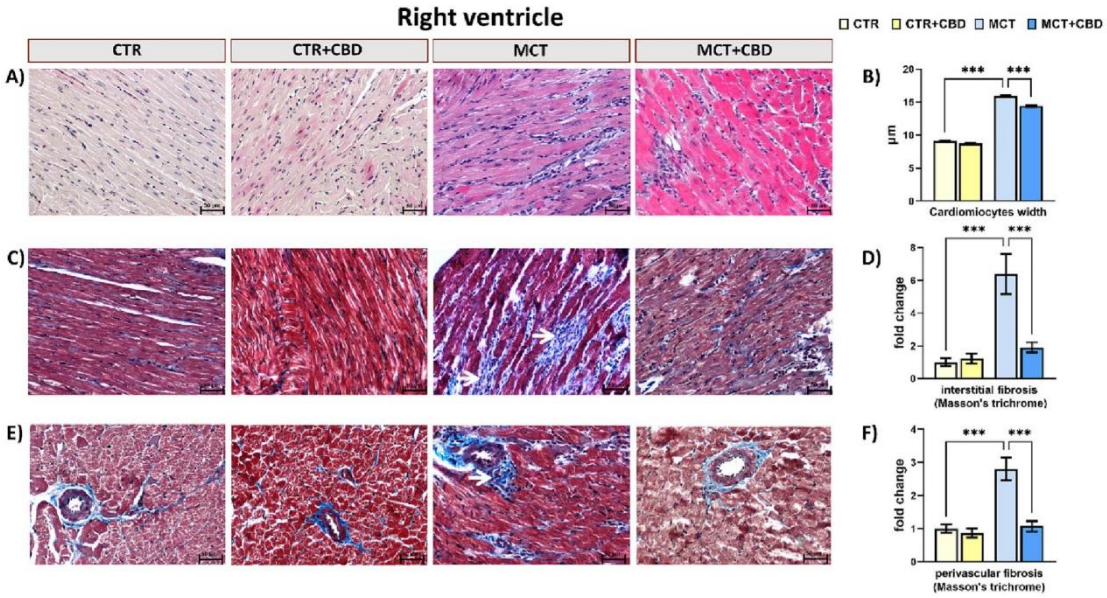

Fig. 3. (A) Representative cardiac sections (H + E staining) and the impact of cannabidiol (CBD) on (B) cardiomyocytes width; (C) representative micrographs of Masson's trichrome staining, interstitial fibrosis; (E) representative micrographs of Masson's trichrome staining, perivascular fibrosis (magnification 200×) and their quantification shown as an area of interstitial fibrosis (D) and perivascular fibrosis (F) in right ventricles from control (CTR) and monocrotaline (MCT)-induced pulmonary hypertension rats. The white arrows on the micrographs indicate the area of fibrosis. All data are shown as mean ± SEM, $n = 6–7$; $***p < 0.001$.

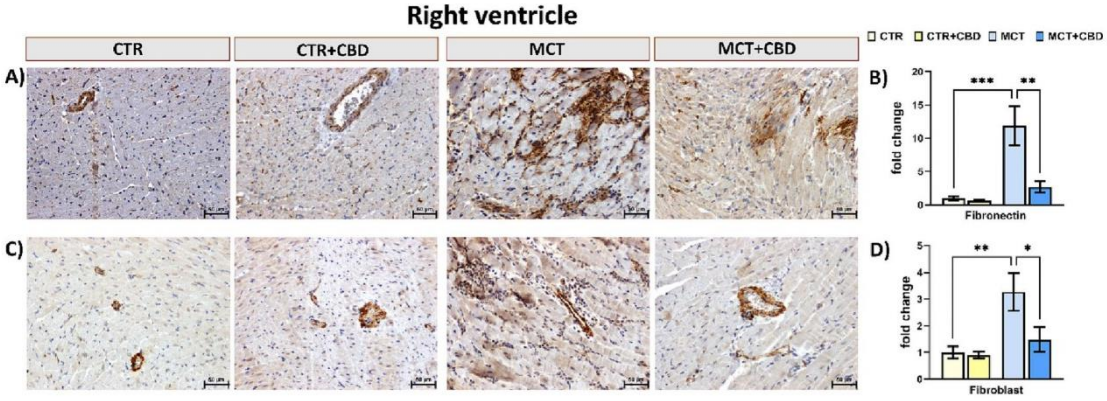

Fig. 4. The impact of cannabidiol (CBD) on (A) fibronectin staining (B) and their quantification; (C) and fibroblast staining (D) and their quantification in rats' right ventricles from control (CTR) and monocrotaline (MCT)-induced pulmonary hypertension rats. The dark brown precipitate represents the intensity of fibroblast and fibronectin staining (magnification 200×). All data are shown as mean ± SEM, $n = 6–7$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pulmonary artery binding Sun et al. [34] observed an increased expression of TGF-β1, SMAD2 and phosphorylated SMAD2. This fact may be in favor of our model of PH, in which a similar shift in the direction of these profibrotic parameters is likely due to overloading the RV, similar to human PAH. Additionally, our study again reveals a special role of the TGF-β1/SMAD2 pathway in RV fibrosis in the MCT-induced PH model in rats. The decrease in VE-cadherin expression in the RV along with the simultaneous increase in TGF-β1, SMAD2, pSMAD2 and α-SMA parameters suggest the involvement of the TGF-β1/SMAD2 pathway in the process of endothelial to mesenchymal transition (EndoMT) and RV remodeling in the MCT-induced PH.

Galectin 3 is considered a marker of myocardial fibrosis in patients with heart failure, and is actively involved in this process. Additionally, Gal-3 is expressed in human left atrial cardiomyocytes and is also associated with fibrosis and atrial fibrillation [35]. Moreover, Fenster et al. [36] suggested that Gal-3 may be a good plasma marker in human PH that correlates with disease progression. Recent studies suggest a close synergistic relationship between Gal-3 and the TGF-β1/SMAD pathway. Exogenously administered Gal-3 enhances the synthesis of TGF-β1, SMAD2 and collagen I in atrial fibroblasts, and exogenously

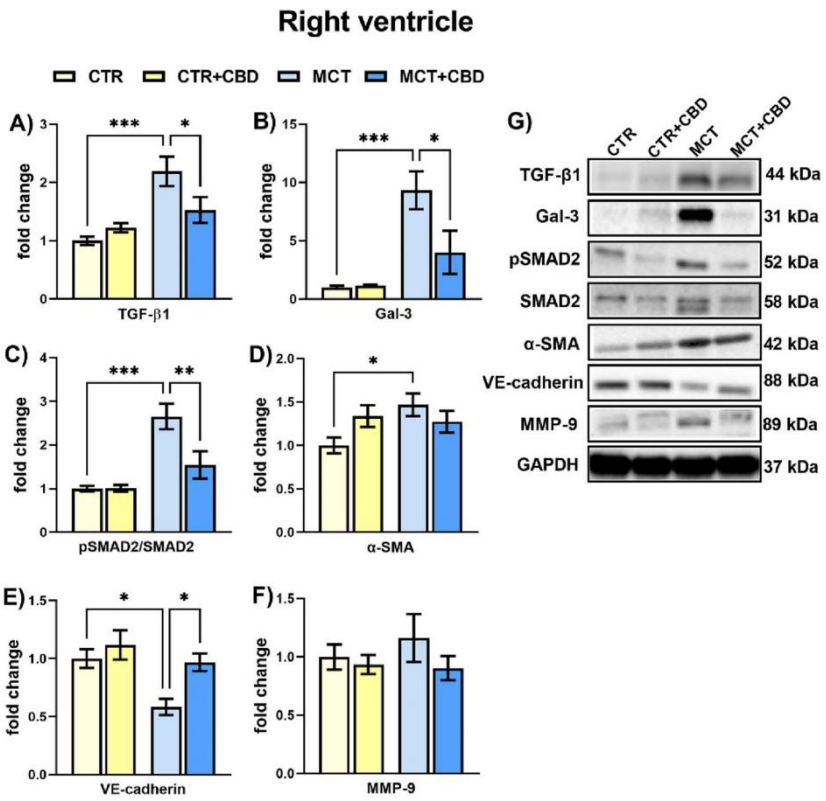

Fig. 5. The impact of cannabidiol (CBD) on the expression of (A) transforming growth factor beta 1 (TGF-β1), (B) galectin 3 (Gal-3), (C) phosphorylated form of suppressor of mothers against decapentaplegic/ suppressor of mothers against decapentaplegic (pSMAD2/SMAD2), (D) alpha-smooth muscle actin (α-SMA), (E) vascular endothelial cadherin (VE-cadherin), (F) matrix metalloproteinase-9 (MMP-9) and (G) their representative bands determined by Western blot in right ventricles from control (CTR) and monocrotaline (MCT)-induced pulmonary hypertension. All data are shown as mean ± SEM, $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

added TGF-β1 enhances the synthesis of Gal-3 [37]. Moreover, Western blot analysis of all four markers mentioned above (TGF-β1, SMAD2, Gal-3 and collagen I) showed higher expression levels in the atria of the heart of patients with rheumatic heart disease with sinus rhythm [37]. Thus, our research suggests that Gal-3 is involved in the process of RV remodeling through its interaction with the TGF-β1/SMAD2 pathway. In line with our results, He et al. [7] demonstrated increased expression of Gal-3 in areas of collagen deposits in the RV in rats with MCT-induced PH and suggested that this may be related to the TGF-β1 pathway.

4.3. Impact of CBD on right ventricle of rats with MCT-induced PH

In our study, we demonstrated for the first time the diminishing effect of chronic CBD administration on one of the most commonly used markers of heart failure today, i.e. NT-proBNP in the plasma of MCT-induced PH rats. This seems to be of great importance since lowering the plasma NT-proBNP level in patients treated with sildenafil and bosentan correlates with the response to therapy in patients with PH [38].

CBD, as mentioned in the introduction and the discussion (General), has a number of beneficial properties limiting the development of PH, and also has an effect on the function of the cardiovascular system. In our study, CBD decreased the width of cardiomyocytes and the area of interstitial and perivascular fibrosis assessed by Masson's trichrome staining and fibronectin, and also reduced the amount of profibrotic markers such as fibroblasts, TGF-β1, Gal-3, SMAD2, pSMAD2 in RV in rats with MCT-induced PH (Fig. 6).The first reports of the anti-fibrotic potential of CBD in mouse model of diabetes-induced cardiac fibrosis appeared in 2010 [23], whereas nearly 10 years later, Vuolo et al. showed that CBD reduces the content of collagen fibers in the airways and alveoli in asthmatic mice [39]. Accordingly, cardiomyocytes width-limiting effect of CBD was observed by Pędzińska-Betiuk et al. [14], who showed that chronic administration of CBD at the same dose to the SHR reduced the width of the cardiomyocytes in the RV compared to the SHR. Our study reports that CBD has the property of reducing ECM deposition in the RV due to the fact that chronic administration of CBD to MCT-induced PH rats reduced the area of interstitial and perivascular fibrosis and the expression of fibroblasts and fibronectin. CBD at a dose of 10 mg/kg and 20 mg/kg reduced the area of fibrosis, and also weakened the expression of profibrotic collagen I deposition and fibronectin genes in mouse model of type I diabetic cardiomyopathy [23]. Chronic administration of CBD at 10 mg/kg for 42 days was also effective in reducing the area of fibrosis and deposition of collagen in the left ventricle in the mouse model of experimental autoimmune myocarditis [19]. There is limited amount of studies trying to elucidate the mechanism of CBD's antifibrotic action, however, existing reports show that non-thiophilic and chemically stable derivative of the CBD quinol (VCE004.8) has the potential to inhibit collagen synthesis in fibroblasts and myofibroblast differentiation and may be effective in preventing skin fibrosis induced by bleomycin in mice [40]. Moreover, one of the potential mechanisms of action of VCE004.8 proposed by the authors was activation of the TGF-β1 signaling pathway [40]. What is more EHP-101 the oral lipid formulation of the new aminoquinone cannabidiol VCE-004.8 prevents and inhibits angiotensin II-induced inflammation and cardiac fibrosis in mice [41].

As previously mentioned, the TGF-β1/SMAD 2 signaling pathway is

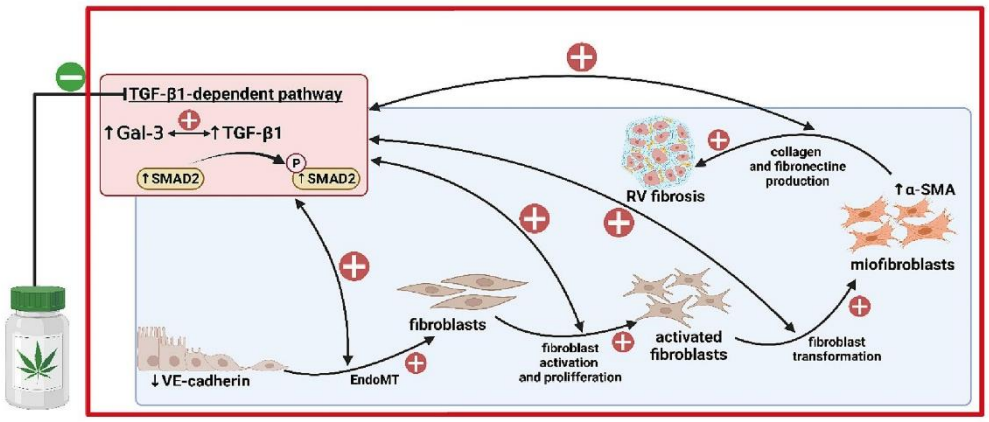

Fig. 6. A summary of the process of RV fibrosis in heart. In the pathological process of heart remodeling, activated fibroblasts transform into myofibroblasts, which in turn produce extracellular matrix (ECMs) such as collagen or fibronectin. Myofibroblasts induce local activation of many profibrotic factors, such as transforming growth factor β1 (TGF-β1), which regulates ECMs deposition via, inter alia, suppressor of mothers against decapentaplegic (SMAD) signaling pathways. TGF-β1 stimulates activation and transformation of fibroblast into myofibroblasts, which is expressed by increased expression of alpha-smooth muscle actin (α-SMA), however, such an effect is possible as a result of the previous SMAD2 phosphorylation [26]. Importantly, SMAD2 is found in plexiform lesions in pulmonary arterial hypertensive (PAH) patients [27]. Endothelial to mesenchymal transition (EndoMT) is a process in which endothelial cells transform into mesenchymal cells or smooth muscles, and the TGF-β1/SMAD pathway plays a key role in this process [5]. TGF-β1 activates endothelial cells to produce fibrogenic mediators and also induces EndoMT in an experimental cardiac fibrosis model. During EndoMT progression, endothelial cells lose endothelial-specific markers, such as vascular endothelial cadherin (VE-cadherin), and acquire mesenchymal fibroblast-like markers, including α-SMA [28]. The tissue architecture is disturbed and fibrous tissue is deposited. A consequence of these processes is excessive stiffening and constriction of the right ventricle and deterioration of its systolic-diastolic function [29]. Created in BioRender.

one of the main inducers of fibrosis in the RV in the MCT-induced PH. Chronic administration of CBD to MCT-induced PH rats decreased the expression of TGF-β1, SMAD2 and pSMAD2. Based on our results chronic CBD administration probably inhibits the TGF-β1/SMAD2 pathway in the RV in the MCT-induced PH, and thus attenuates RV remodeling. Inhibition of the TGF-β1/SMAD pathway resulted in an improvement in cardiac function and reduction of its fibrosis in a rat model of high-salt diet-induced heart fibrosis [42]. As mentioned earlier, there have already been reports of the association of the TGF-β1/SMAD2 pathway with Gal-3 [37]. In our study, the direction of changes in these parameters in the RVs after CBD administration is the same, which confirms that the target point for CBD action may be the TGF-β1/SMAD2 pathway. Additional, inhibition of Gal-3 by CBD in the RV may have a preventive effect in the PH because less RVSP increase and reduced RV remodeling were observed in Gal-3$^{-/-}$ mice exposed to hypoxia compared to wild-type hypoxic mice [43]. In our previous study CBD reduced the expression of TGF-β1 and Gal-3 in the lungs of rats with MCT-induced PH [45]. Remiszewski et al. [46] showed that chronic administration of the classic cannabinoid type 1 receptor (CB_1-R) antagonist (JD5037) reduced RV hypertrophy in MCT-induced PH rats, but there was no effect of CB_1-R antagonist administration on TGF-β1 and Gal-3 expression in the lungs of PH rats. Due to these discrepancies, we hypothesize that in our study CBD reduces the expression of TGF-β1 and Gal-3 through CB_1-R-independent mechanisms, despite the fact that cannabinoid receptors type 1 and 2 ($CB_{1,2}$-Rs) have been reported to correlate with TGF-β1 and/or Gal-3. CB_1-Rs expression in myofibroblast cell culture was increased after TGF-β1 treatment, and Col3a1 expression after TGF-β1 myofibroblast stimulation was attenuated in the presence of a selective CB_1-R antagonist, suggesting some correlation between CB_1-Rs and TGF-β1 [47]. Other studies show that pharmacological blockade or deletion of CB_1-R attenuates TGF-β1 expression in the lung tissue of mice with pulmonary fibrosis [48,49]. Correlations of the CB_2-R with TGF-β1 and Gal-3 are less reported, however administration of a CB_2-R antagonist has been shown to increase Gal-3 [50], TGF-β1 and pSMAD3 expression in mouse skin samples [51]. Nevertheless, Lu et al. [11] did not reveal any significant phenotypic or pathological differences between wild-type and CB_2-R-deficient ($CB2^{-/-}$) PH mice. In the context of PH, it is also worth mentioning that CBD showed a vasorelaxant effect on human pulmonary arteries, and neither CB_1-Rs nor CB_2-Rs were involved in this effect [13]. In conclusion, the current knowledge shows that the $CB_{1,2}$ -Rs are promising targets for drugs in PH, especially by their property to modulate fibrosis, however, it seems that CBD attenuates PH by other mechanisms than by directly affecting these receptors due to its very weak affinity (Ki = 4350 nM for CB_1 -R and Ki = 2399 nM for CB_2-R) [52] which is why we focused on the other mechanisms.

After CBD administration, more VE-cadherin in the RV homogenate compared with MCT group was observed, which suggests that CBD may be involved in the inhibition of EndoMT process. However, CBD only caused a tendency to reduce the α-SMA expression, which means that CBD does not completely inhibit the remodeling process, and although the number of fibroblasts was reduced after chronic administration of CBD, the very process of converting fibroblasts into myofibroblasts may not have been completely inhibited. Additionally, the lack of influence of CBD on the increased Fulton index in rats by MCT may also be an argument for this hypothesis [10].

5. Limitations

CBD was only examined in this study in relation to fibrosis parameters in male rats' RV. Female rats should be included in the research, since PH develops particularly in females [44]. The research could be extended to other PH models in the future. In our study, only one model was used. Moreover, CBD does not completely reverse the changes induced by MCT-induced PH, which may suggest that it does not act on all signaling pathways leading to RV remodeling in PH. In addition, due to the insufficient hemodynamic studies, we can only suggest a role or relationship of the TGF-β1/SMAD2 pathway in attenuating right ventricular fibrosis, and we can only speculate that attenuation of right ventricular fibrosis by CBD may improve right ventricular function.

Based on these data, we can speculate that CBD, by reducing the pressure in the pulmonary circulation, also might reduce the right ventricular systolic pressure. In addition, we hypothesize that CBD improves right ventricular systolic and diastolic function and improves hemodynamic parameters by reducing right ventricular fibrosis through the mechanisms we proposed.

6. Conclusions

In conclusion, chronic CBD administration attenuates the fibrosis of the RV in the MCT-induced PH in rats. We suggest, that the major pathway involved in this process is the TGF-β1/SMAD2 signaling cascade (Fig. 6). Accordingly, we obtained similar results in the lung tissue [45]. Thus, CBD may be used in the future as an adjuvant therapy in the treatment of PH, but this requires confirmation in further experimental and clinical studies, with particular attention to other signaling pathways.

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CRediT authorship contribution statement

Anna Krzyżewska: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Marta Baranowska-Kuczko:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Irena Kasacka:** Investigation, Methodology, Resources, Writing – review & editing. **Hanna Kozłowska:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

No conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2023.166753.

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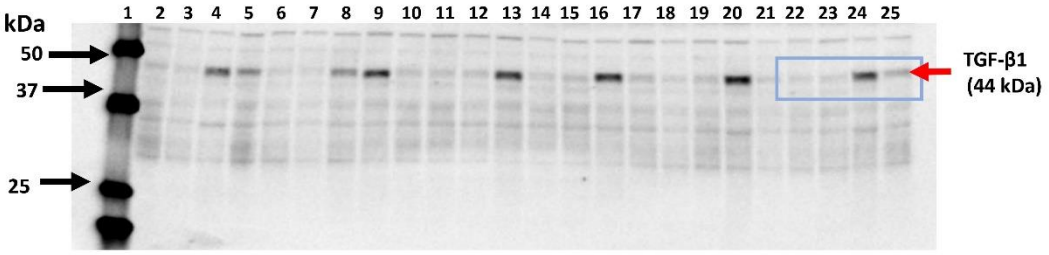
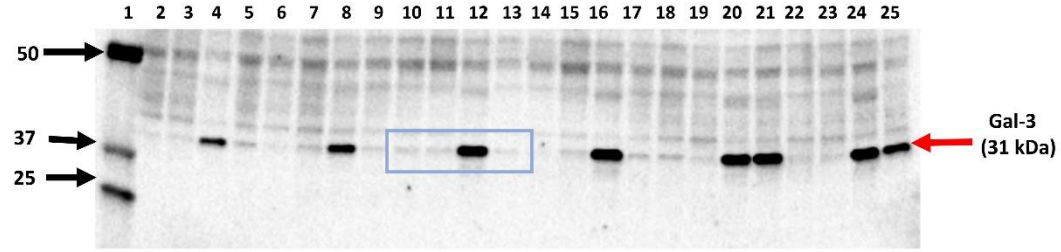
Suplement do drugiej pracy oryginalnej

Right ventricle- TGF-β1, Gal-3

TGF-β1
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

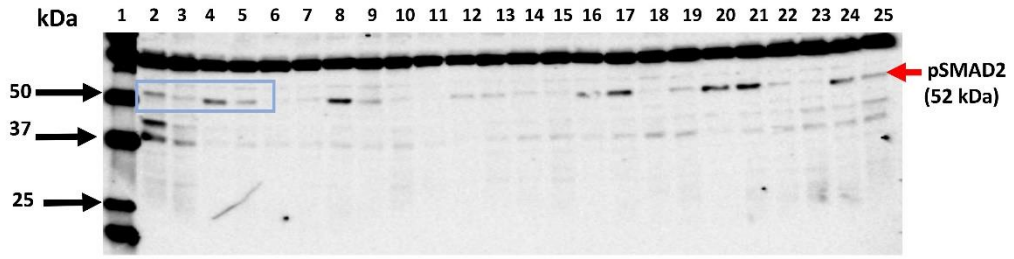


Gal-3
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

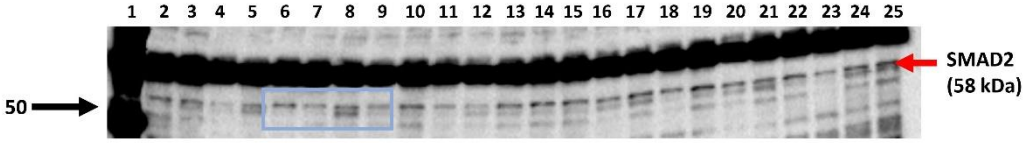


Right ventricle-pSMAD2, SMAD2

pSMAD2
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

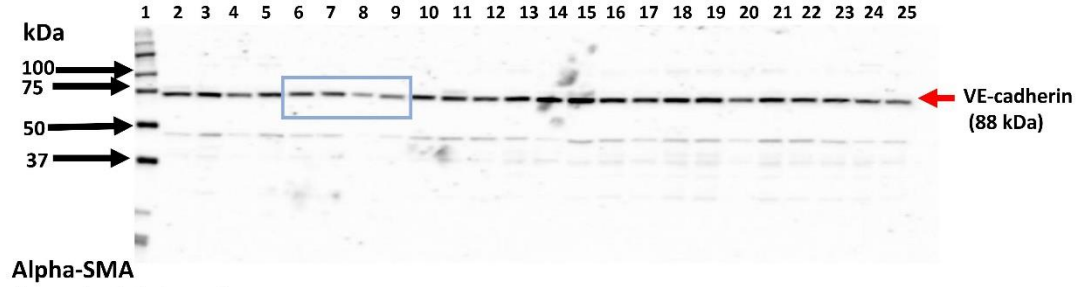


SMAD2
From the left (rows):
standard (1) CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

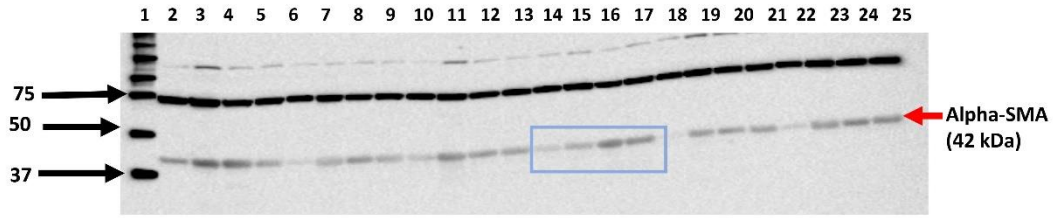


Right ventricle- VE-cadherin, alpha-SMA

VE-cadherin
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

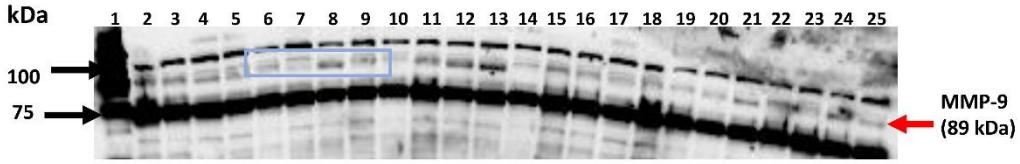


Alpha-SMA
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25

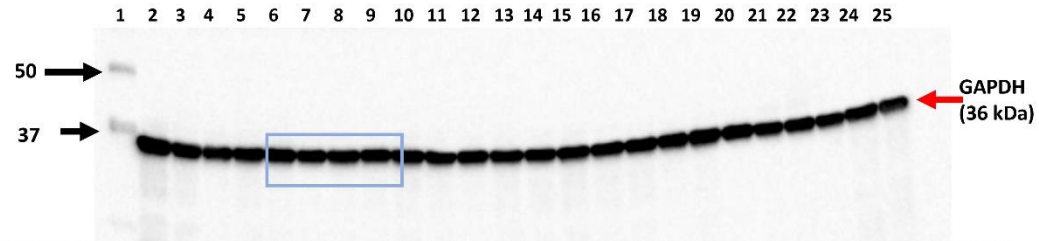


Right ventricle- MMP-9

MMP-9
From the left (rows):
standard (1), CTR: 2, 6, 10, 14, 18, 22; CTR+CBD: 3, 7, 11, 15, 19, 23; MCT: 4, 8, 12, 16, 20,24; MCT+CBD: 5, 9, 13, 17, 21, 25



The levels of the proteins detected were normalized to GAPDH.



The red arrows indicate bands detected with the primary antibody. The black arrows indicate specific molecular weights obtained using the Western C standard which help identify and characterize the molecules separated in a gel. The blue rectangles show bands that have been selected to be shown in Figures in manuscript.

Rozdział 13. Zgoda Lokalnej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach

UCHWAŁA NR 88/2018

z dnia 27.11.2018 r.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Olsztynie

§ 1

Na podstawie art. 48 ust. 1 pkt. 1 / art. 48 ust. 1 pkt. 2[1] ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: **„Kompleksowa ocena potencjalnie protekcyjnego działania kannabidiolu w doświadczalnym modelu tętniczego nadciśnienia płucnego"** z dnia 15.11.2018 r., złożonego przez **Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej (0019)**, adres **ul. Mickiewicza 2D; 15-222 Białystok**[2] zaplanowanego przez **Olga Sadowska**[3], przy udziale[4] (nie dotyczy) Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ[5]

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku **89/2018**.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: **badania podstawowe A, sercowo-naczyniowy układ krążenia krwi i limfy.**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **Dotkliwa.**
3. Doświadczenie będzie przeprowadzane na gatunkach lub grupach gatunków[6]: **140 szt., szczur wędrowny (*Rattus norvegicus*), stado niekrewniacze; Cmdb:Wi, wiek 6-8 tygodni (200 - 250 g), samce.**
4. Doświadczenie będzie przeprowadzane przez: **Olga Sadowska, Hanna Kozłowska, Marta Baranowska- Kuczko.**
5. Doświadczenie miało być przeprowadzane w terminie[7] **07.01.2019 – 15.10.2020**.
6. Doświadczenie będzie przeprowadzone w ośrodku[8]: **nie dotyczy**.
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w: **nie dotyczy.**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ....., w sposób: **nie dotyczy.**

---

[1] Niewłaściwy zapis usunąć
[2] imię i nazwisko oraz adres i miejsce zamieszkania albo nazwę oraz adres i siedzibę użytkownika, który przeprowadzi to doświadczenie, z tym że w przypadku gdy użytkownikiem jest osoba fizyczna wykonująca działalność gospodarczą, zamiast adresu i miejsca zamieszkania tej osoby – adres i miejsce wykonywania działalności, jeżeli są inne niż adres i miejsce zamieszkania tej osoby;
[3] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[4] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[5] Niewłaściwy zapis usunąć
[6] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[7] Nie dłużej niż 5 lat
[8] Podać jeśli jest to inny ośrodek niż użytkownik

9. Doświadczenie **zostanie**[9] poddane ocenie retrospektywnej w terminie do **3 miesięcy** od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.

§ 3

Uzasadnienie: Po dokonaniu oceny wniosku zgodnie z art. 47 ust. 1 i 2 ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266) Lokalna Komisja Etyczna w Olsztynie stwierdza, że projekt nie budzi zastrzeżeń pod względem celowości jego wykonania, liczby użytych zwierząt oraz zasadności i klasyfikacji procedur objętych wnioskiem i wyraża zgodę na przeprowadzenie doświadczenia. Osobą odpowiedzialną za przeprowadzenie badań zgodnie z procedurami opisanymi we wniosku jest **Olga Sadowska**.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

(Pieczęć lokalnej komisji etycznej)

UNIWERSYTET WARMIŃSKO-MAZURSKI
w Olsztynie
LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
10-718 Olsztyn, ul. Oczapowskiego 13/4

Podpis przewodniczącego komisji

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej
do Spraw Doświadczeń na Zwierzętach
prof. dr hab. Jerzy Juśkiewicz

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:

1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje: Osoba planująca doświadczenie; Zespół ds. dobrostanu.

[9] Niewłaściwy zapis usunąć

Rozdział 14. Oświadczenie autora rozprawy doktorskiej

mgr Anna Krzyżewska

Zakład Fizjologii i Patofizjologii Doświadczalnej

Uniwersytet Medyczny w Białymstoku
ul. J. Kilińskiego 1
15-089 Białystok

Białystok, 14.02.2024 r.

Oświadczenie autora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. Krzyżewska A, Baranowska-Kuczko M, Mińczuk K, Kozłowska H. Cannabinoids-A New Perspective in Adjuvant Therapy for Pulmonary Hypertension. Int J Mol Sci. 2021;22(18):10048. doi:10.3390/ijms221810048.

2. Krzyżewska A, Baranowska-Kuczko M, Jastrząb A, Kasacka I, Kozłowska H. Cannabidiol Improves Antioxidant Capacity and Reduces Inflammation in the Lungs of Rats with Monocrotaline-Induced Pulmonary Hypertension. Molecules. 2022;27(10):3327. doi:10.3390/molecules27103327.

3. Krzyżewska A, Baranowska-Kuczko M, Kasacka I, Kozłowska H. Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats. Biochim Biophys Acta Mol Basis Dis. 2023;1869(6):166753. doi:10.1016/j.bbadis.2023.166753.

wchodzących w skład mojej rozprawy doktorskiej polegał na * **opracowaniu koncepcji, metodologii i planu badań, walidacji metod badawczych, wykonaniu części eksperymentalnej, interpretacji wyników, analizie statystycznej, przeglądzie elektronicznych baz publikacji naukowych i zgromadzeniu piśmiennictwa, przygotowaniu manuskryptów, a także pozyskaniu części źródeł finansowania**, co określam jako **60%** udziału w przygotowaniu wyżej wymienionych publikacji.

Anna Krzyżewska
.....
Podpis autora rozprawy doktorskiej

PROFESOR
Zakładu Fizjologii i Patofizjologii
Doświadczalnej
prof. dr hab. Hanna Kozłowska
.....
Podpis promotora

Marta Baranowska Kuczko
.....
Podpis promotora pomocniczego

**W przypadku każdej z włączonych do cyklu prac zaleca się złożenie oświadczenia przez autora wskazujące na jego merytoryczny oraz procentowy wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu autora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej z prac.*

Rozdział 15. Oświadczenia współautorów rozprawy doktorskiej

Prof. dr hab. n. farm. Hanna Kozłowska
.....
imię i nazwisko współautora
Zakład Fizjologii i Patofizjologii Doświadczalnej
.....
nazwa jednostki

Białystok, 14.02.2024 r.
miejscowość, data

Uniwersytet Medyczny w Białymstoku
ul. J. Kilińskiego 1
15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *„Cannabinoids - a new perspective in adjuvant therapy for pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Krzysztof Mińczuk, Hanna Kozłowska,* opublikowanej w *International Journal of Molecular Sciences,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 20% polegał na ustaleniu koncepcji pracy, ocenie merytorycznej pracy, a także analizie i dyskusji danych literaturowych.

2. *„Cannabidiol improves antioxidant capacity and reduces inflammation in the lungs of rats with monocrotaline-induced pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Anna Jastrząb, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Molecules,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 15% polegał na ustaleniu koncepcji badania, analizie i dyskusji wyników oraz ocenie merytorycznej pracy.

3. *„Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Biochimica et Biophysica Acta - Molecular Basis of Disease,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 20% polegał na ustaleniu koncepcji badania, analizie i dyskusji wyników oraz ocenie merytorycznej pracy.

Jednocześnie wyrażam zgodę na wykorzystanie przez **Annę Krzyżewską** publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

.....Hanna Kozłowska.....
Podpis

Dr hab. n. farm. Marta Baranowska-Kuczko
.....
imię i nazwisko współautora

Białystok, 14.02.2024 r.
miejscowość, data

Zakład Fizjologii i Patofizjologii Doświadczalnej/
Zakład Farmacji Klinicznej
.....
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
ul. J. Kilińskiego 1
15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *„Cannabinoids - a new perspective in adjuvant therapy for pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Krzysztof Mińczuk, Hanna Kozłowska,* opublikowanej w *International Journal of Molecular Sciences,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 15% polegał na współuczestnictwie w opracowaniu koncepcji manuskryptu oraz jego edytowaniu i ocenie merytorycznej.
2. *„Cannabidiol improves antioxidant capacity and reduces inflammation in the lungs of rats with monocrotaline-induced pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Anna Jastrząb, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Molecules,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 10% polegał na współuczestnictwie w opracowaniu koncepcji badania, analizie i dyskusji wyników oraz ocenie merytorycznej pracy.
3. *„Cannabidiol allewiates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Biochimica et Biophysica Acta - Molecular Basis of Disease,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 10% polegał na współuczestnictwie w opracowaniu koncepcji badania, analizie i dyskusji wyników oraz ocenie merytorycznej pracy.

Jednocześnie wyrażam zgodę na wykorzystanie przez **Annę Krzyżewską** publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Marta Baranowska-Kuczko
.....
Podpis

Prof. dr hab. n. med. Irena Kasacka
.....
imię i nazwisko współautora

Białystok, 14.02.2024 r.
miejscowość, data

Zakład Histologii i Cytofizjologii
.....
nazwa jednostki

Uniwersytet Medyczny w Białymstoku
ul. J. Kilińskiego 1
15-089 Białystok

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *„Cannabidiol improves antioxidant capacity and reduces inflammation in the lungs of rats with monocrotaline-induced pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Anna Jastrząb, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Molecules,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 10% polegał na oznaczeniu wybranych parametrów stanu zapalnego w tkance płucnej szczurów oraz pomoc w opracowaniu metod badań histologicznych i analizie wyników.

2. *„Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Biochimica et Biophysica Acta - Molecular Basis of Disease,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 10% polegał na oznaczeniu wybranych parametrów włóknienia w prawych komorach serca szczurów oraz pomoc w opracowaniu metod badań histologicznych i analizie wyników.

Jednocześnie wyrażam zgodę na wykorzystanie przez **Annę Krzyżewską** publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

KIEROWNIK
Zakładu Histologii i Cytofizjologii
prof. dr hab. Irena Kasacka
.....
Podpis

Dr n. farm. Krzysztof Mińczuk
.....
imię i nazwisko współautora
Zakład Fizjologii i Patofizjologii Doświadczalnej
.....
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ul. J. Kilińskiego 1
15-089 Białystok

Białystok, 14.02.2024 r.
miejscowość, data

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *„Cannabinoids - a new perspective in adjuvant therapy for pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Krzysztof Mińczuk, Hanna Kozłowska,* opublikowanej w *International Journal of Molecular Sciences,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 5% polegał na współuczestniczeniu w pisaniu i edytowaniu manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie przez **Annę Krzyżewską** publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

.....
Podpis

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Białystok, 14.02.2024 r.
miejscowość, data

Oświadczenie

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. *„Cannabidiol improves antioxidant capacity and reduces inflammation in the lungs of rats with monocrotaline-induced pulmonary hypertension"* autorów *Anna Krzyżewska, Marta Baranowska-Kuczko, Anna Jastrząb, Irena Kasacka, Hanna Kozłowska,* opublikowanej w *Molecules,* wchodzącej w skład rozprawy doktorskiej *„Ocena wpływu kannabidiolu na parametry zapalne i włóknienia w tkance płucnej i prawej komorze serca w szczurzym modelu nadciśnienia płucnego indukowanego monokrotaliną"*, wynoszący 5% polegał na oznaczeniu parametrów stresu oksydacyjnego.

Jednocześnie wyrażam zgodę na wykorzystanie przez **Annę Krzyżewską** publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne.

Anna Stasiewicz
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Podpis

Rozdział 16. Dorobek naukowy

Łączna wartość Impact Factor całego dorobku naukowego: **46.032**

Łączna ilość punktów MNiSW całego dorobku naukowego: **1040**

Wskaźnik Hirscha według Web of Science/Scopus: **3/4**

Cytowania według Web of Science Core Collection: **50**

16.1. Wykaz publikacji stanowiących podstawę rozprawy doktorskiej

1**. Krzyżewska A.,** Baranowska-Kuczko M., Mińczuk K., Kozłowska H.: Cannabinoids-A New Perspective in Adjuvant Therapy for Pulmonary Hypertension. Int J Mol Sci., 2021, 22:10048.

Punktacja IF: **6.208**; MNiSW: **140**

2. **Krzyżewska A.**, Baranowska-Kuczko M., Jastrząb A., Kasacka I., Kozłowska H.: Cannabidiol Improves Antioxidant Capacity and Reduces Inflammation in the Lungs of Rats with Monocrotaline-Induced Pulmonary Hypertension. Molecules., 2022, 27:3327.

Punktacja IF: **4.600**; MNiSW: **140**

3. **Krzyżewska A.**, Baranowska-Kuczko M., Kasacka I., Kozłowska H.: Cannabidiol alleviates right ventricular fibrosis by inhibiting the transforming growth factor β pathway in monocrotaline-induced pulmonary hypertension in rats. Biochim Biophys Acta Mol Basis Dis., 2023, 1869:166753.

Punktacja IF: **6.200**; MNiSW: **140**

16.2. Wykaz innych publikacji naukowych

1. Sadowska O., Baranowska-Kuczko M., Gromotowicz-Popławska A., Biernacki M., Kicman A., Malinowska B., Kasacka I., **Krzyżewska A.,** Kozłowska H.: Cannabidiol Ameliorates Monocrotaline-Induced Pulmonary Hypertension in Rats. Int J Mol Sci. 2020, 21:7077.

Punktacja IF: **5.924**; MNiSW: **140**

2. Mińczuk K., Baranowska-Kuczko M., **Krzyżewska A.**, Schlicker E., Malinowska B.: Cross-Talk between the (Endo)Cannabinoid and Renin-Angiotensin Systems: Basic Evidence and Potential Therapeutic Significance. Int J Mol Sci. 2022, 23:6350.

Punktacja IF: **5.600**; MNiSW: **140**

3. **Krzyżewska A.**, Baranowska-Kuczko M., Kasacka I., Kozłowska H.: Cannabidiol inhibits lung proliferation in monocrotaline-induced pulmonary hypertension in rats. Biomed Pharmacother. 2023, 159:114234.

Punktacja IF: **7.500**; MNiSW: **100**

4. Toczek M., Ryszkiewicz P., Remiszewski P., Schlicker E., **Krzyżewska A.**, Kozłowska H., Malinowska B.: Weak Hypotensive Effect of Chronic Administration of the Dual FAAH/MAGL Inhibitor JZL195 in Spontaneously Hypertensive Rats as Revealed by Area under the Curve Analysis. Int J Mol Sci. 2023, 24:10942.

Punktacja IF: **5.600**; MNiSW: **140**

5. **Krzyżewska A.,** Baranowska-Kuczko M., Galicka A., Kasacka I., Mińczuk K., Kozłowska H.: Cannabidiol may prevent the development of congestive hepatopathy secondary to right ventricular hypertrophy associated with pulmonary hypertension in rats. Pharmacol Rep. 2024.

Punktacja IF: **4.400**; MNiSW: **100**

Autor i współautor dwóch monografii naukowych:

1. **Anna Krzyżewska**, Oksana Litwiniuk, Marta Baranowska-Kuczko, Hanna Kozłowska. Płucne nadciśnienie tętnicze: patofizjologia i terapia. Kierunki rozwoju badań w naukach ścisłych i przyrodniczych. Teoria i praktyka. Red. nauk. Joanna Kotyńska, Monika Naumowicz. Łódź-Kielce: ArchaeGraph, 2021.

Punktacja MNiSW: **20**

2. Marta Baranowska-Kuczko, **Anna Krzyżewska**, Hanna Kozłowska. Kannabidiol - jak wpływa na układ naczyniowy? Kierunki rozwoju badań w naukach ścisłych i przyrodniczych. Teoria i praktyka. Red. nauk. Joanna Kotyńska, Monika Naumowicz. Łódź-Kielce: ArchaeGraph, 2021.

Punktacja MNiSW: **20**

16.3. Komunikaty zjazdowe z konferencji polskich i zagranicznych

1. **Anna Krzyżewska**, Marta Baranowska-Kuczko, Hanna Kozłowska. Evaluation of the anti-inflammatory and anti-proliferative properties of cannabidiol in an experimental model of monocrotalin-induced pulmonary hypertension. Polska, Gdańsk, 2021, poster.

2. Patryk Remiszewski, Anna Pędzińska-Betiuk, Krzysztof Mińczuk, Jolanta Weresa, **Anna Krzyżewska**, Barbara Malinowska. Effects of peripheral cannabinoid cb1 receptor inverse agonist jd5037 in mono- and polytherapy with metformin in a monocrotaline induced rat model of pulmonary arterial hypertension. Polska, Gdańsk, 2021, prezentacja ustna.

3. Patryk Remiszewski, Anna Pędzińska-Betiuk, Krzysztof Mińczuk, Jolanta Weresa, **Anna Krzyżewska**, Barbara Malinowska. Combined AMPK activation and CB1 receptor blockade as a new target in pulmonary arterial hypertension treatment. Estonia, Tallin, 2021, poster.

4. **Anna Krzyżewska**, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska. Evaluation of the effect of chronic administration of cannabidiol on selected markers of inflammation and remodeling in the lungs in rats in a monocrotaline-induced pulmonary hypertension model. Polska, Kazimierz Dolny, 2021, prezentacja ustna.

5. Anna Andruczyk, Marta Baranowska-Kuczko, **Anna Krzyżewska**, Hanna Kozłowska, Irena Kasacka, Barbara Malinowska. Wpływ chronicznego podania kannabidiolu na zmiany ekspresji receptorów kannabinoidowych CB1 i CB2 oraz waniloidowych TRPV1 w naczyniach krwionośnych wyizolowanych od szczurów z nadciśnieniem pierwotnym i wtórnym. Polska, Olsztyn, 2022, poster.

6. **Anna Krzyżewska**, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska. Cannabidiol reduces lung and heart fibrosis in rats with monocrotaline-induced pulmonary hypertension. Hiszpania, Barcelona, 2022, poster.

7. **Anna Krzyżewska**, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska. Cannabidiol alleviates right ventricular fibrosis by inhibiting the TGF-β1/SMAD2 pathway in an experimental monocrotaline-induced pulmonary hypertension model in rats. Polska, Ustroń, 2022, prezentacja ustna.

8. **Anna Krzyżewska**, Krzysztof Mińczuk, Monika Kloza, Marta Baranowska-Kuczko, Hanna Kozłowska. Cannabigerol relaxes the blood vessels of rats with primary hypertension and normotension and lowers blood pressure in normotensive rats. Polska, Żyrardów, 2023, prezentacja ustna.

9. Monika Kloza, **Anna Krzyżewska**, Hanna Kozłowska, Barbara Malinowska, Marta Baranowska-Kuczko. Influence of sodium-glucose cotransporter 2 (SGLT2) inhibitor on

the vascular reactivity in isolated arteries of spontaneously hypertensive rats. Preliminary study. Polska, Łódź, 2023, poster.

10. Marta Baranowska-Kuczko, Monika Kloza, Hanna Kozłowska, **Anna Krzyżewska**, Patryk Remiszewski, Barbara Malinowska. Weight-dependent variability in pulmonary vascular reactivity in the monocrotaline-induced pulmonary hypertension in Sprague-Dawley rats. Preliminary study. Polska, Łódź, 2023, prezentacja ustna.

11. Marta Baranowska-Kuczko, Hanna Kozłowska, Monika Kloza, **Anna Krzyżewska**, Anna Andruczuk, Rafał Rydzewski, Barbara Malinowska. The complex effects of cannabidiol on the vascular tone in animal models of arterial hypertension. Polska, Żyrardów, 2023, prezentacja ustna.

12. **Anna Krzyżewska**, Marta Baranowska-Kuczko, Irena Kasacka, Hanna Kozłowska. Cannabidiol reduces right ventricular fibrosis by regulating TGF-β and β-catenin signaling pathways in monocrotaline-induced pulmonary hypertension in rats. Hiszpania, Barcelona, 2024, poster.

16.4. Granty i projekty naukowe

Kierownik czterech projektów naukowych z subwencji Uniwersytetu Medycznego w Białymstoku:

1. Wpływ kannabidiolu na wybrane parametry stresu oksydacyjnego w tkance płucnej w wywołanym monokrotaliną doświadczalnym modelu nadciśnienia płucnego u szczura, 2021.
2. Wpływ kannabidiolu na wybrane parametry stanu zapalnego w prawej komorze serca w wywołanym monokrotaliną doświadczalnym modelu nadciśnienia płucnego u szczura, 2022.
3. Wpływ kannabidiolu na wybrane parametry układu angiotensynowego w prawej komorze serca w wywołanym monokrotaliną doświadczalnym modelu nadciśnienia płucnego u szczura, 2023.
4. Ocena wazodylatacyjnego działania kannabigerolu w wybranych łożyskach naczyniowych izolowanych od szczurów normotensyjnych oraz szczurów ze spontanicznym nadciśnieniem tętniczym, 2024.

Współwykonawca 16 projektów naukowych finansowanych z Narodowego Centrum Nauki i subwencji Uniwersytetu Medycznego w Białymstoku:

1. Novel combination therapies of pulmonary arterial hypertension targeting blockade of peripheral cannabinoid CB1 receptors plus iNOS inhibition or AMPK activation" (OPUS, 2021 – 2025). Kierownik prof. dr hab. Barbara Malinowska.
2. Wpływ kannabidiolu na wybrane parametry hemostazy w wywołanym monokrotaliną doświadczalnym modelu tętniczego nadciśnienia płucnego u szczura. 2020. Kierownik prof. dr hab. Hanna Kozłowska.
3. Ocena wpływu kannabidiolu na profil ekspresji wybranych genów i białek związanych z układem renina-angiotensyna-aldosteron w naczyniach krwionośnych wyizolowanych od szczurów z nadciśnieniem pierwotnym oraz wtórnym DOCA-salt. 2021. Kierownik dr hab. Marta Baranowska-Kuczko.
4. Czy kannabidiol wpływa na stan zapalny w płucach w doświadczalnym modelu nadciśnienia płucnego u szczura wywołanego alkaloidem roślinnym monokrotaliną? 2021. Kierownik prof. dr hab. Hanna Kozłowska.
5. Interakcja pomiędzy mechanizmami kannabinoidowymi i angiotensynowymi w jądrze przykomorowym podwzgórza a regulacja ciśnienia krwi u szczurów z nadciśnieniem pierwotnym. 2021. Kierownik dr Krzysztof Mińczuk.
6. Ocena wpływu kannabidiolu na profil ekspresji wybranych białek związanych z układem renina-angiotensyna-aldosteron oraz sirtuinami SIRT 1 i SIRT3 w tkankach szczurów z nadciśnieniem płucnym wywołanym monokrotaliną. 2022. Kierownik dr hab. Marta Baranowska-Kuczko.
7. Wpływ kannabidiolu na receptory kannabinoidowe CB1, CB2, waniloidowe TRPV1 oraz jądrowe PPARγ w doświadczalnym modelu nadciśnienia płucnego u szczura wywołanego alkaloidem roślinnym monokrotaliną. 2022. Kierownik prof. dr hab. Hanna Kozłowska.
8. Ocena wpływu obwodowego antagonisty receptorów kannabinoidowych CB1 JD5037 w monoterapii i politerapii z aktywatorem AMPK metforminą na wybrane parametry stresu oksydacyjnego i stanu zapalnego w doświadczalnym modelu tętniczego nadciśnienia indukowanego monokrotaliną u szczurów. 2022. Kierownik Prof. dr hab. Barbara Malinowska.
9. Zależna od wieku rola sirtuin 1 i 3 (SIRT1 i SIRT3) i dysfunkcji śródbłonka w tętnicach płucnych i aortach szczurów z nadciśnieniem płucnym wyindukowanym monokrotaliną. 2023. Kierownik dr hab. Marta Baranowska-Kuczko.

10. Ocena czynności skurczowej oraz zmian morfologicznych i biochemicznych w izolowanych lewych przedsionkach i mięśniach brodawkowatych lewej komory serca szczura z nadciśnieniem płucnym wywołanym monokrotaliną. 2023. Kierownik dr Anna Pędzińska-Betiuk.
11. Wpływ kannabidiolu na zmiany w wątrobie w doświadczalnym modelu nadciśnienia płucnego u szczura wywołanego alkaloidem roślinnym monokrotaliną. 2023. Kierownik prof. dr hab. Hanna Kozłowska.
12. Ocena zależnego od wieku rozwoju nadciśnienia płucnego indukowanego monokrotaliną u szczurów. 2023. Kierownik Prof. dr hab. Barbara Malinowska.
13. Wpływ wieku szczurów Wistar pochodzących z Centrum Medycyny Doświadczalnej na rozwój nadciśnienia płucnego i w porównaniu ze szczurami Sprague-Dawley. 2024. Kierownik Prof. dr hab. Barbara Malinowska.
14. Ocena działania roślinnego kannabinoidu - kannabigerolu na czynność skurczowo-rozkurczową izolowanej tętnicy płucnej podsegmentarnej człowieka. 2024. Kierownik prof. dr hab. Hanna Kozłowska.
15. Ocena kurczliwości oraz zmian biochemicznych i histologicznych w izolowanych mięśniach brodawkowatych prawej komory serca szczura z nadciśnieniem płucnym indukowanym sugenem i hipoksją. 2024. Kierownik dr Anna Pędzińska-Betiuk.
16. Ocena wpływu białka fuzyjnego receptora aktywiny typu IIA-Fc (ActRIIA-Fc) na modulację układu sercowo-naczyniowego w modelach in vitro. 2024. Kierownik dr hab. Marta Baranowska-Kuczko.

16.5. Stypendia i nagrody

1. Nagroda główna za najlepszą prezentację ustną wygłoszoną podczas XXV Sympozjum Sekcji Kardiologii Doświadczalnej Polskiego Towarzystwa Kardiologicznego w Kazimierzu Dolnym, 2021.

2. Stypendium z własnego funduszu stypendialnego Uniwersytetu Medycznego w Białymstoku 2022/2023 za uzyskanie wyróżniających wyników w nauce oraz osiągnięcia naukowe.

3. Wyróżnienie w postaci grantu edukacyjnego za abstrakt zgłoszony na Światowe Sympozjum Nadciśnienia Płucnego (Stowarzyszenie Nadciśnienia Płucnego, Hiszpania, Barcelona, 2024).

16.6. Wyjazdy

1. Wyjazd do Królestwa Niderlandów w ramach programu Erasmus+: trzymiesięczny staż w VU University Medical Center (Amsterdam UMC, locatie VUmc), Department of Physiology (lipiec 2023 – wrzesień 2023).

16.7. Wykaz innych aktywności naukowych, popularyzacyjnych i organizacyjnych

1. Recenzent publikacji naukowych w czasopismach: Adv Ther. (2023, IF: 3.8) i Biomed Pharmacother. (2024, IF: 7.5).

2. Opiekun naukowy trzech prac magisterskich na kierunku Farmacja na Wydziale Farmaceutycznym z Odziałem Medycyny Laboratoryjnej Uniwersytetu Medycznego w Białymstoku w latach 2022 - 2024.

4. Członek Polskiego Towarzystwa Kardiologicznego.

5. Opiekun pomocniczy Koła Naukowego prowadzonego przy Zakładzie Fizjologii i Patofizjologii Doświadczalnej, Uniwersytet Medyczny w Białymstoku.

6. Współorganizator zajęć z dziećmi i młodzieżą na Podlaskim Festiwalu Nauki i Sztuki, Uniwersytetu Medycznego w Białymstoku. Temat zajęć: Podejmij wyzwanie: Policz swoje zmysły, 2022.

7. Współorganizator pokazu przedstawionego podczas 26. Pikniku Naukowego Polskiego Radia i Centrum Nauki Kopernik w Warszawie. Temat pokazu: Złap oddech. Spirometria kiedyś i dziś, 2023.