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Evaluation of the potential therapeutic significance of chronically administered cannabinoids in experimental models of arterial and pulmonary hypertension in rats

Doctoral dissertation
based on a series of scientific publications
in the field of medical and health sciences
in the discipline of pharmacology and pharmacy

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Chapter 1. List of publications constituting the doctoral dissertation

1. **Review paper**: Remiszewski P.; Malinowska B. Why multitarget vasodilatory (endo)cannabinoids are not effective as antihypertensive compounds after chronic administration: Comparison of their effects on systemic and pulmonary hypertension. *Pharmaceuticals* **2022**, 15, 1119, doi: 10.3390/ph15091119. (IF) = 5.215, (MES) = 100 pts
2. **Original paper 1**: Remiszewski P.; Jarocka-Karpowicz I.; Biernacki M.; Jastrząb A.; Schlicker E.; Toczek M.; Harasim-Symbor E.; Pędzińska-Betiuk A.; Malinowska B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. *Int J Mol Sci* **2020**, 21, 1295, doi:10.3390/ijms21041295. (IF) = 5.924, (MES) = 140 pts
3. **Original paper 2**: Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K.; Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. *Front Pharmacol* **2022**, 13, 965613, doi: 10.3389/fphar.2022.965613. (IF) = 5.988, (MES) = 100 pts

Total Impact Factor (IF) for the series of publications: 17.127

Total Ministry of Education and Science (MES) points for the series of publications: 340

Chapter 2. List of abbreviations

2-AG – 2-arachidonoylglycerol

AEA – anandamide

AMPK – 5' adenosine monophosphate-activated protein kinase

BP – blood pressure

CB_1R – cannabinoid type 1 receptor

CB_2R – cannabinoid type 2 receptor

CBD – cannabidiol

CTR – control animals

DBP – diastolic blood pressure

DOCA – deoxycorticosterone acetate

$dP/dt_{min/max}$ – the rate of rise/decrease of right ventricular pressure

FAAH – fatty acid amide hydrolase

Gal-3 – galectin-3

HR – heart rate

i.p. – intraperitoneally

LV + S – left ventricle + septum

MAGL – monoacylglycerol lipase

MCT – monocrotaline

MET – metformin

O_2 – oxygen

PAH – pulmonary arterial hypertension

PH – pulmonary hypertension

PPAR – peroxisome proliferator-activated receptor

PUFAs – polyunsaturated fatty acids

RV – right ventricle

RVSP – right ventricular systolic pressure

SBP – systolic blood pressure

SHAM – sham-operated animals

SHR – spontaneously hypertensive rat

TGFβ1 – transforming growth factor β1

THC – Δ^9-tetrahydrocannabinol

TRPV1 – transient receptor potential vanilloid type 1

WKY – Wistar-Kyoto rat

Chapter 3. Introduction

Arterial (systemic) and pulmonary hypertension are multi-factorial, high-pressure disorders. The first one is a civilizational disease, the second one is characterized by a very high mortality rate. Treatment resistance cases and low effectiveness makes exploring new therapeutic strategies an extremely vital mission. Among many others, (endo)cannabinoids recognized for their strong vasorelaxant properties are suggested as possible drugs for various types of hypertension.

In my doctoral dissertation I will refer to the relevant sections, tables and figures of the original paper 1 (Remiszewski et al., 2020), the original paper 2 (Remiszewski et al., 2022) and a review paper (Remiszewski and Malinowska, 2022).

Arterial hypertension

Arterial hypertension has been characterized more precisely in section 2 of the review paper. Briefly, it is a multifunctional disease, which main outcome is an increase in blood pressure (BP) over 140 mmHg (systolic; SBP) and 80 mmHg (diastolic; DBP). Regarding the cause of hypertension development, two main types are introduced: primary (essential) hypertension, which accounts for up to 95% of cases but with no one and clear origin, and secondary hypertension with identified, other disease-related genesis (Ott and Schmieder, 2022). The pathophysiological basis of arterial hypertension is multifaceted and consists of the interplay between renal, humoral, vascular and central mechanisms, that normally maintain physiological BP, but their failure eventually leads to disease progression. With more than one billion people suffering from hypertension (30-45% of adults worldwide), this condition is considered the most critical and expensive public health problem and the leading single changeable contributor to global all-cause mortality and disability (Oparil et al., 2018). A wide range of available treatment options cover most of the diagnosed patients, however up to 20% of cases are classified as treatment-resistant (Brant et al., 2022). Drugs focusing on novel mechanisms are therefore anticipated.

Pulmonary hypertension

Pulmonary hypertension (PH) is a rare disease with elevated pressure (>20 mmHg) and resistance in the pulmonary circulation. Among the 5 groups of PH, the least frequent and most intensively studied is pulmonary arterial hypertension (PAH)

(Hassoun, 2021). Compared to arterial hypertension or even to other groups of PH, PAH is an extremely rare (ca. 51 cases per million) condition. The greatest problem with PAH lies in its high mortality. The average survival of patients receiving pharmacotherapy is about 7 years (Bisserier et al., 2020). Such unfavourable statistics are, on one hand, due to the complex pathophysiology of the disease including vasculopathy and following heart failure (Hassoun, 2021), but on the other hand, are connected with very limited therapeutic options, covering mostly pulmonary vasorelaxation (Sommer et al., 2021). Lung and heart inflammation and fibrosis, as contributing factors in the pathobiology of PH, might become mechanisms of action of explored innovative treatment strategies (Sitbon et al., 2019; Prisco et al., 2020). Therefore, the search for novel potential drug targets is extremely significant in the case of PAH. Nowadays, a gold standard of care in PAH is an early combined therapy with at least two compounds (Ruopp and Cockrill, 2022). A more detailed description of PH can be found in section 3 of the review article and the original article 2. In my original paper 2, I used the terms PAH and PH in the case of human and animal studies, respectively, which is a recommended practice.

Experimental models of hypertension

Research on pathophysiology and potential therapeutic options would not be possible without experimental models of hypertension (Dignam et al., 2022; Jama et al., 2022). As shown in Table 1 of the review paper, there are many models, which reflect primary or secondary arterial hypertension. Among them, the main focus should be placed on the spontaneously hypertensive rat (SHR) (primary) and deoxycorticosterone acetate (DOCA)-salt (secondary) models, which were used in the original paper 1. Pulmonary hypertension is also represented by a couple of models, including a monocrotaline (MCT)-induced one at the forefront, which was introduced in the original paper 2, but also others, like a hypoxia-induced model with its modification by additional sugen administration.

Cannabinoids and the endocannabinoid system

Cannabinoids are the group of compounds first isolated from *Cannabis sativa*. There are 3 main classes of cannabinoids, depending on their origin:

1) phytocannabinoids – both natural and synthetic compounds having their archetype in the plant, e.g. Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD);

2) synthetic cannabinoids – synthetic molecules, mostly agonists of cannabinoid receptors, e.g. CP-55,940, WIN 55,212-2;
3) endocannabinoids and endocannabinoid-like compounds – produced and acting endogenously, e.g. anandamide (AEA), 2-arachidonoylglycerol (2-AG).

All 3 classes of cannabinoids can affect the endocannabinoid system, which, besides endocannabinoids, is formed with cannabinoid receptors: both classical (cannabinoid type 1 (CB_1R) and type 2 (CB_2R)) and non-classical (transient receptor potential vanilloid type 1, TRPV1; GPR18; GPR55; peroxisome proliferator-activated receptors, PPAR), enzymes responsible for endocannabinoids synthesis and degradation (e.g. fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MAGL responsible for the metabolism of AEA and 2-AG, respectively) or transmembrane transporters. A number of compounds, mostly synthetic, act through interaction (other than receptor activation) with elements of the endocannabinoid system, e.g. URB597, which inhibits FAAH or JD5037, the peripheral antagonist of CB_1R. Both pro- and anti-hypertensive effects of the sensitive to cannabinoids receptors activation can be found in Figure 1 and section 6 of the review paper.

Promising results demonstrate the strong, tension-dependent vasodilatory effects of (endo)cannabinoids in isolated resistance arteries or pulmonary arteries (including human) (see section 7 and Table 2 of the review paper) and the involvement of the endocannabinoid tone in the cardiovascular system regulation in hypertension (Toczek and Malinowska, 2018). Moreover, numerous studies show the impact of (endo)cannabinoids on the cardiovascular system after their acute administration (described in detail in section 8 of the review article). The main outcomes include different responses in anesthetized and conscious animals. In anesthetized rats, a triphasic BP response occurs, with prolonged CB_1R-dependent hypotension in phase III. The effect is stronger in hypertensive individuals. Conscious experiments resulted however in an increase in BP, a mechanism of which is complex, not fully-identified but with the participation of CB_1Rs (Malinowska et al., 2012).

All of these together suggest potential therapeutic beneficial effects of (endo)cannabinoids in different types of hypertension. Nevertheless, only experiments with the chronic administration of these compounds allow for the verification of the above theory.

Many proposed indications supported by studies and a few registered drugs (Epidiolex, Sativex) are proof of the high potential of CBD as a therapeutic solution. It may also become a strategy for the treatment of cardiovascular diseases, including hypertension (Kicman and Toczek, 2020) not only because of its vasodilatory (Baranowska-Kuczko et al., 2020) but also anti-inflammatory (Sunda and Arowolo, 2020) and anti-oxidant (Pereira et al., 2021) properties. So far, BP-reducing effects of CBD were observed under stress conditions in humans (Sultan et al., 2020) and in stressed animals (Granjeiro et al., 2011). However, the effect of CBD on the BP of hypertensive individuals has been studied in one study only, where in conscious SHR a single intraperitoneal (*i.p.*) dose of CBD failed to diminish BP (Kossakowski et al., 2019).

Since peripheral overactivity of CB_1Rs induces cardiac, pulmonary, liver and kidney fibrosis and promotes inflammation and/or oxidative stress (Puhl, 2020; Kicman et al., 2021), their blockade could become a potential therapeutic strategy (Cinar et al., 2020). Knocking out CB_1R or chronic administration of their peripheral antagonists (AM6545 or JD5037) diminishes inflammation and fibrosis and enhances animal survival in many experimental models. Increased fibrosis and inflammation of the vasculature and cardiac tissue are important causes/results of PH development. This is why CB_1R blockade is being suggested as the target of PH therapy. Using a peripherally restricted antagonist of CB_1R allows for avoiding central side effects observed previously with rimonabant, which was the reason for its withdrawal (Cinar et al., 2020). Metformin, an activator of 5' adenosine monophosphate-activated protein kinase (AMPK), was found effective in PH not only in animal-based experiments but also in clinical trials (Brittain et al., 2020). Taking into account that the gold standard for PAH treatment nowadays is an early combined therapy with compounds that affect different pharmacological targets (Klinger et al., 2019; Sommer et al., 2021; Tettey et al., 2021; Ruopp and Cockrill, 2022), merging CB_1R antagonism with other molecular pathways (e.g. AMPK activation) is the desired option. It is even more interesting since new compounds with both of these properties were recently introduced (Cinar et al., 2020).

Chapter 4. The aim of the dissertation with the justification of the undertaken research topic

Arterial (systemic) and pulmonary hypertension are complex, high-pressure conditions. Arterial hypertension is a civilizational disease, whereas PH is of concern because of its high mortality rate. Treatment resistance cases and low effectiveness makes studying novel therapies an extremely essential task. Among many others, (endo)cannabinoids known for their strong vasodilatory activity are proposed as potential drugs for a variety of types of hypertension. However, chronic experiments are still needed to evaluate the significance of (endo)cannabinoids in these indications.

CBD is a multi-targeted phytocannabinoid with proven anti-oxidant and anti-inflammatory activity. It has been proposed as a potential anti-hypertensive drug also thanks to its direct vasodilatory activity. Although, since acute administration of CBD in anesthetized SHR caused no alteration of BP, assessment of its chronic influence in various models of experimental hypertension is required to verify its hypotensive action.

Early polytherapy or combined therapy, where therapeutic agents with more than one pathway or pharmacological target are used, is nowadays a gold standard in the treatment of PH. CB_1R antagonism may become useful in PH since it leads to a decrease in fibrosis and inflammation in many pathological states. Combining it with an AMPK activator, metformin, which has been proved effective in experimental models and clinical trials of PAH, not only fulfils the requirement of operating on several targets. It is also the beginning of research on a new group of the third-generation CB_1R antagonists with properties that combine these two mechanisms of action.

Thus, **the aim of my doctoral dissertation was to determine:**

1. the influence of chronic administration of CBD on BP and heart rate (HR) in rats with primary and secondary hypertension and its influence on the redox system, the endocannabinoid system and levels of polyunsaturated fatty acids (PUFAs) in the heart and plasma of rats;
2. the influence of separate and combined administration of the peripheral CB_1R antagonist JD5037 and the AMPK activator metformin on the MCT-induced PH in rats;

3. the effects of chronic administration of (endo)cannabinoids on BP in various models of systemic and pulmonary hypertension (based on available literature).

Chapter 5. Realization of scientific aims, material and methods, summary of research results and discussion

Material and methods

All surgical procedures and experimental protocols were approved by the Local Ethics Committee for Animal Experiments in Olsztyn (resolutions no. 80/2017, 74/2020, 9/WNP/WDO/2021 and 39/WNP/2021).

The experiments were conducted on the following hypertension models:

I. Arterial hypertension:

1. Spontaneously hypertensive rat (SHR) (primary/essential) with Wistar-Kyoto (WKY) control group. The SHR model was used as the most frequently studied genetic hypertensive model.
2. Deoxycorticosterone acetate (DOCA)-salt (secondary) with Wistar (SHAM) control group. The DOCA-salt model was introduced because a salt-rich diet is one of the main lifestyle modifiable factors leading to hypertension.

II. Pulmonary hypertension:

3. Monocrotaline (MCT)-induced with Wistar (CTR) control group. The MCT-induced model is considered the most accepted preclinical rodent model of established PH (also used for the development of PAH-targeted therapies).

Detailed information on the models and their development can be found in the original papers 1 (SHR and DOCA-salt) and 2 (MCT), and in the review paper.

Following compounds and their vehicles were used chronically in the experimental protocol:

- **arterial hypertension:** CBD, 10 mg/kg body weight, *i.p.*, once daily for 14 days
- **pulmonary hypertension***:* JD5037, 3 mg/kg body weight, orally, once daily for 21 days and metformin, 100 mg/kg body weight, orally, once daily for 21 days. JD5037 and metformin were administered separately or in combination.

The rationale for the doses used can be found in the original papers 1 and 2.

Experimental protocols

I. Arterial hypertension

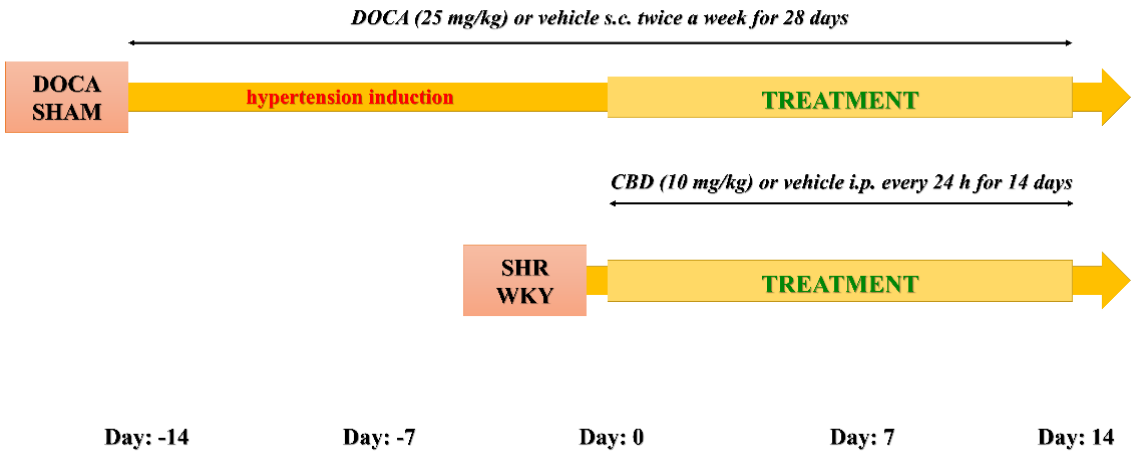

Figure 1. A simplified scheme of experiments on arterial hypertension using a timeline. CBD – cannabidiol; DOCA – deoxycorticosterone acetate; SHAM – sham-operated animals; SHR – spontaneously hypertensive rat; WKY – Wistar-Kyoto rat.

At the beginning of the experiments (day 0), BP (SBP as well as HR) was measured using the non-invasive tail-cuff method to confirm hypertension development (rats with SBP ≥ 150 mmHg were considered hypertensive). After the measurement, rats were randomly assigned to appropriate groups and the first dose of CBD or its vehicle was administered. The tail-cuff method-based determination of cardiovascular parameters was repeated after 7 and 14 days of the treatment.

In another subgroup of animals, SBP, DBP and HR were constantly measured telemetrically in SHR and WKY. This method allows us to record even the smallest, both acute and chronic, changes in those physiological parameters in freely-moving animals.

Body weight was measured daily, before CBD or its vehicle administration.

Twenty-four hours after the last dose of CBD or its vehicle rats were anesthetized with pentobarbitone sodium (300 μmoL/kg; *i.p.*) to collect blood and the heart. The following parameters were determined in the tissues:

- endocannabinoids and endocannabinoid-like compounds;
- FAAH and MAGL activity;
- CBRs expression;

- anti-oxidant enzyme activity and non-enzymatic anti-oxidants level;
- oxidative protein and lipid modifications;
- fatty acids.

II. Pulmonary hypertension

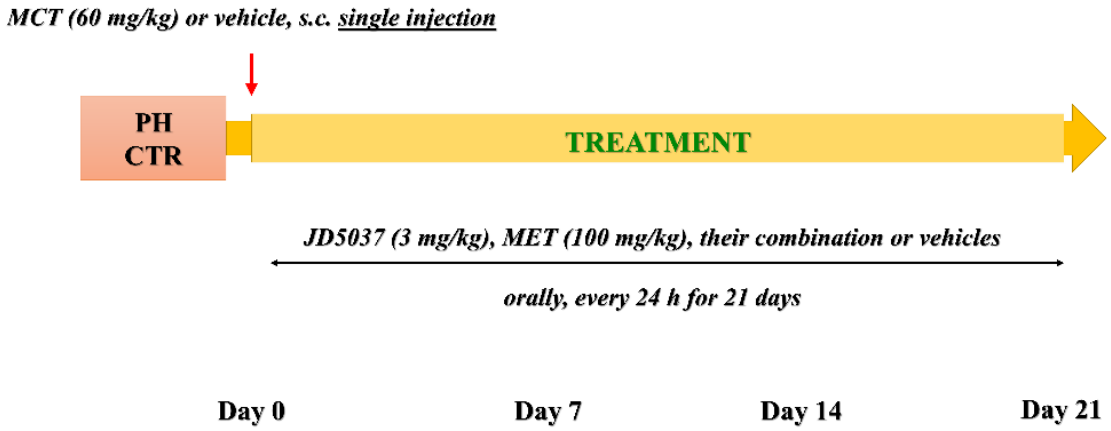

Figure 2. A simplified scheme of experiments on pulmonary hypertension using a timeline. CTR – control group; MET – metformin; MCT – monocrotaline; PH – pulmonary hypertension.

After a single injection of MCT or its vehicle on day 0, SBP was measured non-invasively the next day, before the first dose of tested compounds (JD5037 and/or metformin) or their vehicles were administered. Body weight was measured daily, before compounds or their vehicles administration. After 21 days of treatment, 24 hours after the last dose (on day 22), blood glucose was measured from the lateral tail vein and animals were anesthetized with ketamine and xylazine (*i.p.*, 1 mL of ketamine 100 mg/mL + 100 µL of xylazine 20 mg/mL; 300 µL per 250 g of body weight). After induction of anesthesia rectal temperature was determined and then a pressure catheter with a sensor for the measurement of the right ventricular systolic pressure (RVSP), HR and the rate of rise/decrease of right ventricular pressure ($dP/dt_{min/max}$) was pushed forward through the right jugular vein and placed in the right ventricle (RV). At the same time, blood oxygen level and HR were assessed using a pulse oximeter. After RVSP determination, the heart and lungs were removed. Next, RV, left ventricle with the septum (LV + S), right and left atria, left lung and left kidney were separated and weighed. RV hypertrophy was expressed in two ways: as Fulton's index which is RV weight to LV + S weight (RV/LV + S) and as right ventricular hypertrophy index, which is RV weight to body

weight of the animal. Lung hypertrophy index was expressed as left lung weight to body weight of the animal. Except for hypertrophy indexes, the following parameters were determined in the tissues:

- histopathological scoring and Masson's trichrome staining in the heart;
- pulmonary artery vascular wall thickness and alpha smooth muscle actin immunohistochemical staining in lungs;
- remodelling markers' expression in lungs.

A detailed description of the procedures and statistical analysis of the results can be found in the original papers 1 and 2.

Summary of research results and discussion

The aim of my dissertation was an evaluation of the influence of chronically administered (1) CBD in primary and secondary arterial hypertension and (2) the peripheral CB_1R antagonist JD5037 alone or combined with the AMPK activator metformin in pulmonary hypertension. Additionally, I have reviewed available literature on (endo)cannabinoids in systemic and pulmonary hypertension, which helped me to indicate if those compounds can be used as effective anti-hypertensive drugs.

First of all, I have proven that the used models do reflect arterial and pulmonary hypertension. In SHR and DOCA-salt significant increases in SBP occurred (to the mean value of about 170 mmHg in both models; in both tail-cuff and telemetric method in SHR). In the MCT-induced model I have induced mild or early-stage PH with increased RVSP, Fulton's index, oxygen saturation and changes in the pulmonary vasculature and cardiac tissue (see Figure 4). Occurrence of mild PH results from relatively moderate values of RVSP and lack of many changes characteristic of the end-stage condition, like high mortality or intense RV fibrosis (lack of collagen I increase and only small increase of early predictors of fibrosis – transforming growth factor β1 (TGFβ1) and galectin-3 (Gal-3)). Since lowering the PAH threshold arose (Simonneau et al., 2019), performing experiments and clinical trials on individuals with lower pressure is very much awaited. It should be remembered, that all presently used therapies have been studied in cases with advanced PH and it is unclear if currently available treatment schedules will be helpful for early-diagnosed patients (Stewart et al., 2020).

Most importantly, for the first time, I have shown model- and tissue-dependent changes in endocannabinoids and/or endocannabinoid-like compounds concentrations in systemic hypertension. As shown in Figure 2 and Figure 3 of original paper 1, in addition to AEA and 2-AG, which have been described previously (Biernacki et al., 2018), also six other endocannabinoid-like compounds' levels decreased (or tended to decrease) in the heart of SHR whereas in the heart of DOCA not only 2-AG but also five others increased. On the rule, changes in plasma endocannabinoid levels were similar (but less pronounced).

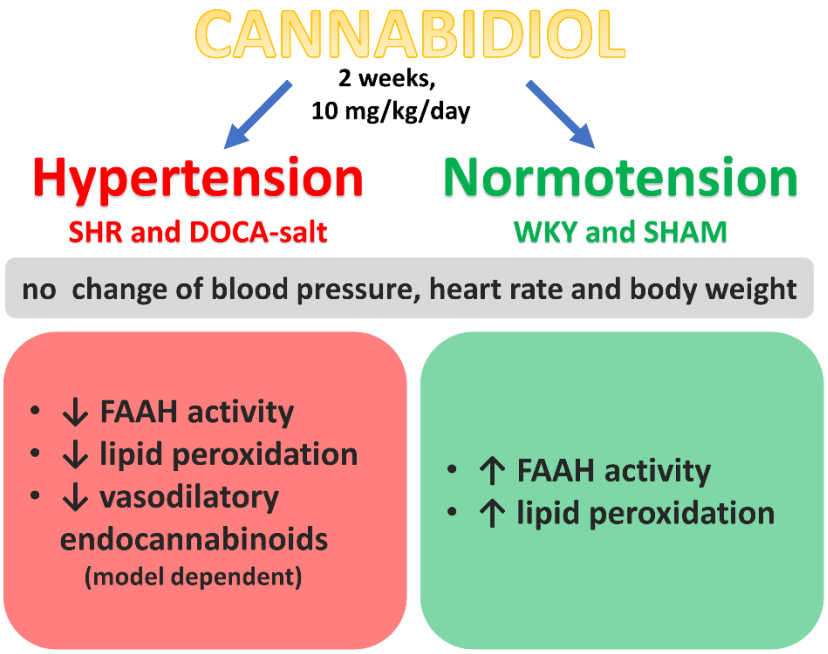

Figure 3. Summary of the most important effects of chronic administration of CBD in models of primary (SHR) and secondary (DOCA-salt) arterial hypertension. DOCA – deoxycorticosterone acetate; FAAH – fatty acid amide hydrolase; SHAM – sham-operated animal; SHR – spontaneously hypertensive rat; WKY – Wistar-Kyoto rat.

As shown in Figure 3, summarizing results that I have obtained in the original paper 1, in arterial hypertension I have not observed the effect of 2-week chronic administration of CBD on BP, HR or body weight. There were, however, some alterations to other parameters. CBD decreased FAAH activity (as described previously, Leishman et al., 2018), diminished lipid peroxidation (but also some anti-oxidant markers) and lowered model-dependently levels of endocannabinoids with vasodilatory properties. Interestingly, in normotension CBD acted oppositely on FAAH activity and lipid

peroxidation (increase); the mechanism of both needs further study. Especially the second activity requires carefulness while using CBD in other indications.

The lack of anti-hypertensive activity of CBD can be explained by its contrary effects on BP, observed not only in my experiments (original paper 1) but also in other authors' studies (Figure 2 in the review paper). First of all, CBD possesses proven vasodilatory activity on systemic vessels, also extracted from hypertensive individuals (Baranowska-Kuczko et al., 2020). What is more, its chronic administration mostly improves the vasodilatory response of arteries (Baranowska-Kuczko et al., 2021) and has protective properties on cardiac function (Pędzińska-Betiuk et al., 2021) (original paper 1). On the other hand, CBD was found to have peripheral sympathomimetic activity (Kossakowski et al., 2019), which together with its partial pro-oxidant (drops in levels of anti-oxidant vitamins) (original paper 1) and pro-inflammatory (increase of pro-inflammatory enzyme level) activity (Baranowska-Kuczko et al., 2021) connected with the ability to decrease vasodilatory endocannabinoids (original paper 1 and Figure 2 of the review paper) leads to failure in lowering BP.

Interestingly, CBD was effective as an anti-PH drug, with anti-inflammatory, anti-oxidant, anti-hypertrophic and vasodilatory properties (Sadowska et al., 2020; Lu et al., 2021; Krzyżewska et al., 2022). The superiority of CBD in pulmonary over systemic hypertension may result from (1) scheme of administration – in arterial hypertension only therapeutic while in PH both therapeutic and preventive were used; (2) differences in systemic and pulmonary arteries reactivity to CBD; or (3) smaller role of the sympathomimetic activity of CBD in the pulmonary circulation (for details, see review paper).

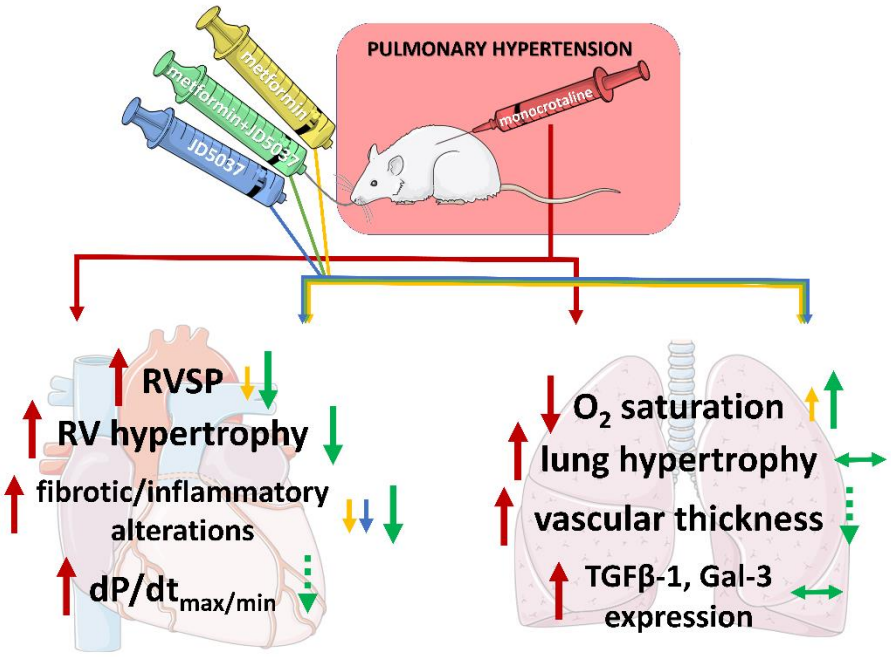

Figure 4. Summary of the most important cardiac (left part) and pulmonary (right part) effects of chronic administration of JD5037, metformin or their combination in an MCT-induced model of pulmonary hypertension. $dP/dt_{max/min}$ – derivatives of RV pressure over time; Gal-3 – galectin-3; MCT – monocrotaline; O_2 – oxygen; RV – right ventricle; RVSP – right ventricular systolic pressure; TGFβ1 – transforming growth factor β1. *Figure prepared using Servier Medical Art*.

As shown in Figure 4, summarizing results that I have obtained in the original paper 2, monotherapy with JD5037 resulted in partial reverse of fibrotic/inflammatory alterations in cardiac tissue. Metformin alone possessed similar activity but it additionally decreased RVSP and increased oxygen saturation. The strongest activity was, although, observed when JD5037 was combined with metformin. Combined therapy decreased RVSP, RV hypertrophy and increased blood oxygen saturation. It also tended to reduce pulmonary vascular thickness. Taking into account, that I performed experiments on mild (or early-stage) PH, where changes related to the disease are not severe, obtaining improvement of such crucial parameters shows the high potential of this treatment.

The reason for the superior effect of combination therapy against most parameters of MCT-induced PH may be that two complementary mechanisms act in parallel. However, a potential additive effect of both compounds is conceivable as well since JD5037 (and other CB_1R antagonists) may act also via activation of AMPK (Liu et al., 2019).

One should keep in mind that other results may be obtained if 1) experimental PH is induced by other stimuli, e.g., sugen plus hypoxia; 2) a therapeutic rather than a preventive paradigm is used; 3) compounds are administered at a higher dose or for a longer time; 4) the end-stage PH is studied (obtained by a longer period of PH development, the use of younger rats and/or another strain, e.g., Sprague-Dawley); and 5) the PH is induced in female animals since in humans it occurs predominantly in women (Hester et al., 2019).

In my review paper, I have compiled all available publications on chronic (endo)cannabinoids administration in arterial (31 papers) and pulmonary hypertension (6 papers). Effects of the therapies were analyzed according to Dr Page Mosaic Theory of hypertension development, where the most relevant mechanisms that may contribute to pathophysiology but also become targets of different types of hypertension treatment occurs (Harrison et al., 2021). Among them, the most crucial are vascular and cardiac effects, inflammation, redox balance and endocannabinoid system involvement (see Figure 2 and Figure 3 of the review paper).

My original research matched results obtained by other authors. The most striking finding of the review paper is the difference in the action of the (endo)cannabinoids based on their target specificity. Multitarget compounds were not effective as anti-hypertensive drugs in arterial hypertension, since they induce responses leading to both a decrease and an increase in BP. My original paper 1 showed such activity in the case of CBD. In PH, both multi- and monotarget (endo)cannabinoids were found effective. One of the examples is JD5037 (especially in combination with metformin). Thus, target-specific therapy in PH may be further studied using third-generation CB_1R antagonists with additional AMPK activation activity.

In summary, my original research both with the literature review showed, that future experiments regarding (endo)cannabinoids in hypertension should be primarily focused on monotarget (or target-specific) compounds in systemic hypertension, but both mono- and multitarget molecules can be tested in pulmonary hypertension.

Chapter 6. Conclusions

1. Contrary anti- and pro-hypertensive effects of chronic CBD administration result in failure of BP decrease in experimental models of primary and secondary hypertension in rats.
2. Chronic 21-day combined administration of metformin and JD5037 attenuated most of the mild PH-induced cardiopulmonary alterations and tended to be more efficient than any of the monotherapies alone.
3. Monotarget (or target-specific) but not multitarget (endo)cannabinoids are effective and should be further studied as anti-hypertensive drugs in systemic hypertension, but both of these groups can be successfully used in PH.

Chapter 7. References

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Chapter 8. Summary in English

Arterial (systemic) and pulmonary hypertension (PH) are multi-factorial, high-pressure disorders. The first one is a civilizational disease, the second one is characterized by a very high mortality rate. Among many others, (endo)cannabinoids recognized for their strong vasorelaxant properties are suggested as possible drugs for various types of hypertension.

The aim of my dissertation was an evaluation of the influence of chronically administered (1) cannabidiol (CBD) in primary and secondary arterial hypertension and (2) the peripheral CB_1R antagonist JD5037 alone or combined with the AMPK activator metformin in PH. Additionally, I have reviewed available literature on (endo)cannabinoids in systemic and pulmonary hypertension, which helped me to determine if those compounds can be used as effective anti-hypertensive drugs.

I have performed all experiments on two rat models of systemic hypertension: spontaneously hypertensive rat (SHR; primary) and deoxycorticosterone acetate (DOCA)-salt (secondary) model, and rat monocrotaline (MCT)-induced model of PH. In arterial hypertension, I have not observed the effect of 2-week chronic administration of CBD on blood pressure (BP), heart rate or body weight. There were, however, some alterations to other parameters. In hypertensive animals, CBD decreased fatty acid amide hydrolase (FAAH) activity, diminished lipid peroxidation and lowered model-dependently levels of endocannabinoids with vasodilatory properties. What is more, in normotension, it increased lipid peroxidation and acted as an FAAH activator.

In PH, monotherapy with JD5037 resulted in partial reverse of fibrotic/inflammatory alterations in cardiac tissue. Metformin alone possessed similar effects, but it also decreased right ventricular systolic pressure (RVSP) and increased oxygen saturation. The strongest activity was, however, observed when JD5037 was combined with metformin. Combined therapy decreased RVSP, right ventricle hypertrophy and increased blood oxygen saturation. It also tended to reduce pulmonary vascular thickness.

The most striking finding of the review paper is the difference in the action of the (endo)cannabinoids based on their target specificity. Multitarget compounds were not effective as anti-hypertensive drugs in arterial hypertension, since they induce responses

leading to both a decrease and an increase in BP. In PH, both multi- and monotarget (endo)cannabinoids were found effective.

In summary, my original research both with literature review showed that: (1) contrary anti- and pro-hypertensive effects of chronic CBD administration result in failure of BP decrease in experimental models of primary and secondary hypertension in rats; (2) chronic 21-day combined administration of metformin and JD5037 attenuated most of the mild PH-induced cardiopulmonary alterations and tended to be more efficient than any of the monotherapies alone; and (3) monotarget (or target-specific) but not multitarget (endo)cannabinoids are effective and should be further studied as anti-hypertensive drugs in systemic hypertension, but both of these groups can be successfully used in PH.

Summary in Polish

Nadciśnienie tętnicze (systemowe) i nadciśnienie płucne (PH) to wieloczynnikowe schorzenia związane z podwyższonym ciśnieniem. Pierwsze to powszechna choroba cywilizacyjna, drugie zaś charakteryzuje się bardzo wysoką śmiertelnością. Sugeruje się, że (endo)kannabinoidy, znane ze swoich silnych właściwości naczyniorozszerzających, mogą stanowić potencjalne leki na różne rodzaje nadciśnienia.

Celem mojej pracy była ocena wpływu przewlekle podawanych: (1) kannabidiolu (CBD) w pierwotnym i wtórnym nadciśnieniu tętniczym oraz (2) obwodowego antagonisty receptorów kannabinoidowych CB_1, JD5037, samego lub w połączeniu z aktywatorem AMPK, metforminą, w PH. Dodatkowo dokonałem przeglądu dostępnej literatury dotyczącej (endo)kannabinoidów w nadciśnieniu systemowym i płucnym, co umożliwiło mi ocenę, czy związki te mogą być stosowane jako skuteczne leki przeciwnadciśnieniowe.

Wszystkie eksperymenty przeprowadziłem na szczurzych modelach eksperymentalnych, t.j. dwóch modelach nadciśnienia systemowego: spontanicznego (SHR; odpowiadającego ludzkiemu nadciśnieniu pierwotnemu) i indukowanego octanem deoksykortykosteronu i wysokosolną dietą (DOCA-salt; nadciśnienie wtórne), a także na modelu PH wywołanym monokrotaliną (MCT). W nadciśnieniu tętniczym nie zaobserwowałem wpływu 14-dniowego przewlekłego podawania CBD na ciśnienie krwi (BP), tętno czy masę ciała. Zauważyłem jednak pewne zmiany innych parametrów. U zwierząt z nadciśnieniem CBD zmniejszył aktywność hydrolazy amidowej kwasów tłuszczowych (FAAH), peroksydację lipidów i obniżył, zależnie od modelu, poziomy endokannabinoidów o właściwościach rozszerzających naczynia krwionośne. W normotensji, odwrotnie, zwiększał on peroksydację lipidów i aktywował FAAH.

W PH monoterapia z użyciem JD5037 spowodowała częściowe odwrócenie zmian zwłóknieniowych/zapalnych w tkance serca. Sama metformina wykazywała podobną aktywność, ale dodatkowo obniżała ciśnienie prawokomorowe w sercu (RVSP) i zwiększała wysycenie krwi tlenem. Najsilniejszą aktywność zaobserwowałem jednak, gdy JD5037 podawany był w połączeniu z metforminą. 21-dniowa terapia skojarzona obniżała RVSP, przerost prawej komory i zwiększała saturację krwi. Wykazywała również tendencję do zmniejszania grubości naczyń płucnych.

Najważniejszym obserwacją z przeglądu literatury jest natomiast różnica w działaniu (endo)kannabinoidów w oparciu o ich specyficzność związaną z punktami uchwytu. Związki o wielu punktach uchwytu (tzw. multitarget) nie były skuteczne jako leki przeciwnadciśnieniowe w nadciśnieniu tętniczym, ponieważ indukują odpowiedzi prowadzące zarówno do obniżenia, jak i wzrostu BP. W PH skuteczne okazały się zarówno (endo)kannabinoidy o wielu, jak i o pojedynczych i/lub sprecyzowanych (tzn. monotarget) mechanizmach działania.

Podsumowując, moje badania oryginalne oraz przegląd literatury wykazały, że: (1) przeciwstawne działanie przeciw- i pro-nadciśnieniowe przewlekle podawanego CBD skutkuje niepowodzeniem obniżenia BP w eksperymentalnych modelach pierwotnego i wtórnego nadciśnienia tętniczego u szczurów; (2) przewlekłe skojarzone podawanie metforminy i JD5037 osłabiało większość łagodnych zmian sercowo-płucnych wywoływanych przez PH i było bardziej skuteczne niż którakolwiek z monoterapii; oraz (3) (endo)kannabinoidy o pojedynczym/sprecyzowanym mechanizmie działania, ale nie te o wielu punktach uchwytu są skuteczne i powinny być dalej badane jako potencjalne leki przeciwnadciśnieniowe w nadciśnieniu systemowym, ale obie te grupy mogą być z powodzeniem stosowane w PH.

Chapter 9.

pharmaceuticals

MDPI

Review

Why Multitarget Vasodilatory (Endo)cannabinoids Are Not Effective as Antihypertensive Compounds after Chronic Administration: Comparison of Their Effects on Systemic and Pulmonary Hypertension

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Abstract: Systemic and pulmonary hypertension are multifactorial, high-pressure diseases. The first one is a civilizational condition, and the second one is characterized by a very high mortality rate. Searching for new therapeutic strategies is still an important task. (Endo)cannabinoids, known for their strong vasodilatory properties, have been proposed as possible drugs for different types of hypertension. Unfortunately, our review, in which we summarized all publications found in the PubMed database regarding chronic administration of (endo)cannabinoids in experimental models of systemic and pulmonary hypertension, does not confirm any encouraging suggestions, being based mainly on in vitro and acute in vivo experiments. We considered vasodilator or blood pressure (BP) responses and cardioprotective, anti-oxidative, and the anti-inflammatory effects of particular compounds and their influence on the endocannabinoid system. We found that multitarget (endo)cannabinoids failed to modify higher BP in systemic hypertension since they induced responses leading to decreased and increased BP. In contrast, multitarget cannabidiol and monotarget ligands effectively treated pulmonary and systemic hypertension, respectively. To summarize, based on the available literature, only (endo)cannabinoids with a defined site of action are recommended as potential antihypertensive compounds in systemic hypertension, whereas both mono- and multitarget compounds may be effective in pulmonary hypertension.

Keywords: (endo)cannabinoids; systemic hypertension; pulmonary hypertension

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1. Introduction

Systemic and pulmonary hypertension are multi-factorial, high-pressure diseases that influence the left and right parts of the heart, respectively. The first one is a civilizational condition that affects one out of every three adults worldwide. The second one impacts only a fraction per thousand of the population but has a very high mortality rate. Treatment resistance and low effectiveness make searching for new therapeutic strategies an important task. Among many others, (endo)cannabinoids are proposed as a possible drug for different types of hypertension. In this review, we inspected this thesis.

2. Systemic Hypertension

Systemic arterial hypertension, commonly known as hypertension, is a multifunctional disease characterized by persistently increased blood pressure (BP) in the systemic arteries, with values over 140 mmHg for systolic BP (SBP) and over 90 mmHg for diastolic BP (DBP) [1–3]. Most cases of hypertension (90–95%) are classified as primary or essential hypertension with a multifactorial genetic–environmental etiology. The remaining cases are those with identified causes (e.g., renal artery stenosis, pheochromocytoma, adrenal

adenoma, or single-gene mutations), known as secondary hypertension [1,3]. Among the main risk factors connected to primary hypertension, many (high sodium and low potassium intake, alcohol consumption, lack of physical activity, overweight and obesity, unhealthy diet, and smoking) can be altered by patients [4].

Estimates show that more than 1.3 billion people (around 30% of adults) suffer from hypertension worldwide. In some countries where the threshold of hypertension has been lowered to ≥130/80 mmHg (e.g., the USA and China), the prevalence increased to about 45% of the adult population [4]. Hence, it should be no surprise that this disease is considered the most critical and expensive public health problem and is the leading single modifiable contributor to all-cause mortality and disability worldwide, responsible for more than 9 million deaths annually [1,3]. Even a small decrease in elevated BP can significantly reduce the risk of major adverse cardiovascular events and death [2].

The pathophysiological basis of hypertension is complex and consists of the interplay between renal, humoral, vascular, and central mechanisms that normally maintain physiological BP, but their malfunction or disruption eventually leads to elevated cardiac output, body fluid volume, and/or peripheral resistance [1,5]. Aside from the predominant significance of enhanced sympathetic tone in the development and progression of hypertension [6], one of the most crucial components of its pathogenesis is the renal renin–angiotensin–aldosterone system (RAAS), which regulates BP by mediating sodium retention, natriuresis, and vasoconstriction [7]. In addition, the vasculature of patients with hypertension is less responsive to vasodilatation and may be remodeled, stiffened, and affected by inflammatory and oxidative changes [8].

The basic first-line treatment of hypertension is based on three main pathways and includes (1) angiotensin-converting enzyme inhibitors, (2) angiotensin receptor antagonists, (3) calcium channel blockers, and (4) diuretics. It is recommended that therapy for hypertension should be carried out, even started, as combined therapy with two or more substances acting by different mechanisms. To provide individualized therapy, other groups are often added to the primary groups, such as β-blockers, mineralocorticoid antagonists, α-blockers, α_2-agonists, direct vasodilators, or renin inhibitors [2]. Despite the wide selection of antihypertensive drugs, there are still around 10–20% cases of treatment-resistant hypertension associated with a higher impact on cardiovascular risk [9] and cases where proper treatment cannot be administered due to the unacceptable side effects of currently available therapies. Drugs directed at novel mechanisms are therefore being sought [1].

3. Pulmonary Hypertension

Pulmonary hypertension (PH) is a rare progressive cardiopulmonary disease characterized by increased pulmonary arterial pressure, which leads to right heart failure and, consequently, premature death. For many years, PH has been defined as mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg. Population studies have shown that the average mPAP in healthy individuals is about 14 mmHg and rarely exceeds 19 mmHg [10]. Elevated pulmonary pressure, up to 19–25 mmHg, increases mortality and the further risk of developing full-blown PH [11–15]. The search for a borderline between "normal" and elevated pressure in pulmonary circulation led, in 2018 [14], to a proposal for a new frontier of the PH of mPAP ≥ 20 mmHg (i.e., two standard deviations above mean pressure) obtained with right heart catheterization. Further hemodynamic classification into pre-capillary PH, isolated post-capillary PH, or combined pre- and post-capillary PH is carried out using values of pulmonary vascular resistance (PVR) and pulmonary arterial wedge pressure (PAWP) [16].

Classification of PH is based on similar histology and pathophysiology but also concurrent treatment strategies and responses to them [13]. The World Health Organization lists five clinical groups: (1) pulmonary arterial hypertension (PAH); (2) PH related to left-sided heart disease; (3) chronic lung disease-related PH; (4) chronic thromboembolic PH; (5) other types of PH [17]. Groups 2 and 3 are the most common (millions of patients worldwide); however, the greatest emphasis is placed on the rarest types, i.e., groups 1

and 4 [16,18]. The epidemiology of PAH is not easy to determine precisely, but currently available data allow us to estimate its incidence at around 5.8 and prevalence at around 51 cases per million [19]. It should be kept in mind that these statistics were made according to the 2003 PH/PAH definition, and the values will probably increase by up to 10% after the mPAP threshold is lowered [20]. The greatest problem with PAH, however, is still high mortality. With the absence of treatment, the average survival of patients in the 1990s was 2.8 years, whereas, with pharmacological intervention, it is now about 7 years [21]. Survival rates are also connected to patient risk profiles. At baseline, the 1-year, 3-year, and 5-year survival rates are approximately 98, 90, and 80% in the low-risk group, 87, 68, and 52% in the intermediate-risk group, and 75, 52, and 33% in the high-risk group, respectively [22,23]. Even though PAH may be caused by well-known factors, such as toxins and drugs (e.g., methamphetamine), HIV infection, schistosomiasis, connective tissue disease, or congenital heart disease, most cases (up to 67%) are of unknown origin (idiopathic) [24].

The pathophysiology of PAH is complex and primarily connected to the vascular remodeling of the three layers of the small distal pulmonary arteries, which results in their obliteration, muscularization, and the formation of characteristic plexiform lesions. All of those changes led to progressive narrowing of blood vessels and increased mPAP and PVR (all cases of PAH are hemodynamically classified as pre-capillary with PVR $\geq$ 3 Wood units) [15,18,24]. Vascular and perivascular inflammation and fibrosis play important roles in the process [25]. As the vessel's changes progress, the right part of the heart must take on an increasing burden. The right ventricle (RV) undergoes hypertrophy, dilatation, fibrosis, inflammation, ischemia, and metabolic disturbances. In the initial phase, RV remodeling remains adaptive with preserved hemodynamic function; however, at some point, it can no longer keep up with the vasculopathy and transforms into a maladaptive phenotype [18,26].

Currently, specific treatment is available mostly for PH groups 1 and 4. In PAH, three main regulatory pathways are the targets of therapy focused on vasodilatation of pulmonary arteries only: (1) nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway with phosphodiesterase type 5 (PDE5) inhibitors (sildenafil, tadalafil) and a soluble guanyl cyclase (sGC) stimulator (riociguat); (2) prostacyclin (PGI_2)-cyclic adenosine monophosphate (cAMP) pathway with PGI_2 analogs (epoprostenol, treprostinil, iloprost) and receptor agonist (selexipag); and (3) endothelin receptor pathway with its antagonists (bosentan, macitentan, ambrisentan) [27]. Most patients with PAH receive more than one drug as up-front combination therapy, which is now the standard of care [15,16,18]. However, none of the currently available therapeutic options can cure PAH, and life expectancy, despite significantly increasing in recent years, is unsatisfactory. Moreover, PAH is a multifactorial disease, and pulmonary vasoconstriction as the primary target of current therapies seems deficient. Therefore, the search for new potential drug targets is extremely important in the case of PAH.

4. Animal Models of Hypertension

Clinical trials and meta-analyses are the most valuable sources of knowledge about the most efficient treatment strategies for every kind of hypertension. However, animal models are needed for preclinical studies to discover the specific genetic, cellular, and molecular mechanisms underlying the disease or to test novel therapeutic strategies. As the human pathophysiology of hypertension differs among individuals, it is difficult to create a model that ideally mimics all disturbances [28–30].

Among animal models of systemic hypertension, there are two main groups. Models based on genetic alterations (both mono- and polygenic), which are closest to essential human hypertension, and those in which hypertension is induced by the researcher's interventions (dietary, pharmacological, and/or surgical), corresponding to secondary hypertension. The most important models of hypertension covered in this review are presented in Table 1. The most frequent model is spontaneously hypertensive rats (SHR); another polygenic model is Dahl salt-sensitive rats. Induced models are most often repre-

sented in publications by three types: angiotensin II (Ang-II), L-N^G-nitro arginine methyl ester (L-NAME, the inhibitor of nitric oxide synthase (NOS)), and deoxycorticosterone acetate (DOCA)-salt models [28,29]. In addition to the most widely used models, many others that reflect some features of hypertension can be found, such as TGR(mRen2)27, in which overexpression of the renin gene is induced, adrenal regeneration hypertension (ARH), in which contralateral adrenal enucleation is performed, and metacorticoid hypertension, which is similar to DOCA-salt but with more stable development of hypertension or renal hypertension (so-called two-kidney, one clip (2K1C), where the renal artery is constricted [29,31,32]. New methods are continuously being developed. For example, recently, two new models of rapid induction of multifactorial heart disease associated with hypertension (SHR and 2K1C), hypothyroidism, and a high-fat diet were introduced [30].

Table 1. Short list of characteristics of chosen models of systemic and pulmonary hypertension.

Type of Hypertension		Model	Main Characteristics
Systemic	**Primary**	SHR	- early age development (starting from 3–4 weeks) - ↑sympathetic activity - RAAS overactivation - ↑arterial wall stiffness - immune alterations
		Dahl salt-sensitive rat	- low-renin hypertension - kidney injury - ↓responses to vasorelaxants and ↑to vasoconstrictors
		TGR(mRen2)27	- suppression of RAAS with high prorenin levels
	Secondary	Ang-II	- RAAS-dependent hypertension - overactivity of the sympathetic nervous system - BP-independent kidney injury - vascular pressor/remodeling activity
		L-NAME	- NOS-deficient hypertension - systemic and renal vasoconstriction - renal interstitial fibrosis and glomerulosclerosis - immune alterations
		DOCA-salt	- low-renin hypertension - suppression of RAAS - severe renal and cardiac complications - remodeled aortic wall - ↑inflammatory signaling
		ARH	- potassium depletion - electrolyte disturbances - renal deficiency
		metacorticoid hypertension	- similar to DOCA-salt - more stable hypertension development
		renal hypertension (2K1C)	- RAAS overactivation
Pulmonary		MCT	- pulmonary vascular damage - remodeling and ↑vascular resistance - RV failure - intense perivascular inflammation - parenchymal alterations - no plexiform lesions

Table 1. *Cont.*

Type of Hypertension	Model	Main Characteristics
	hypoxia	- pulmonary vascular remodeling - RV hypertrophy - absence of RV failure - enhanced pulmonary vasoconstriction - no plexiform lesions
	sugen/hypoxia	- PH more stable than in hypoxia model - presence of RV failure - with plexiform lesions

For respective references, see Section 4. ↑ increase; ↓ decrease; 2K1C—two-kidney, one clip; Ang-II—angiotensin II; ARH—adrenal regeneration hypertension; DOCA—deoxycorticosterone acetate; L-NAME—L-N^G-nitro arginine methyl ester; MCT—monocrotaline; NOS—nitric oxide synthase; PH—pulmonary hypertension; RAAS—renin-angiotensin-aldosterone system; RV—right ventricle; SHR—spontaneously hypertensive rat.

Similar to systemic hypertension, no single animal PH model is likely to be universally appropriate. The classical models are the ones in which PH is induced by the administration of alkaloid, monocrotaline (MCT), or chronic hypoxia. However, the direct toxic effects of MCT on various organs, including the liver and heart, represent a serious limitation of the MCT model [33–35]. Exposure to chronic hypoxic conditions leads to the induction of PH, similar to many PH-causing conditions in humans (e.g., chronic obstructive pulmonary disease). Additional administration of vascular endothelial growth factor (VEGF) receptor antagonist (Sugen) results in severe and irreversible changes (in rats, but not mice) [28,34,36]. In addition to the classic models of PH, more attention is paid to models with a genetic basis, including monogenic ones [37].

5. Cannabinoids as a Potential New Therapy against Systemic and Pulmonary Hypertension

As mentioned in the previous sections, there is still a need for new effective pharmacotherapy against both systemic and pulmonary hypertension. In recent years, scientists, physicians, and patients have paid increasing attention to (endo)cannabinoids, including medical marijuana, since the therapeutic potential of the endocannabinoid system is enormous and is based on all groups of cannabinoids. Thousands of scientific papers, hundreds of clinical trials, and a few approved drugs (Sativex, Marinol, Syndros, Cesamet, and Epidiolex) provide proof of this potential [38–44]. Moreover, one of the potential targets of cannabinoid-based therapy is the cardiovascular system, including systemic and pulmonary hypertension, as was stated in reviews over the last few years [45–57]. Such promising conclusions are based mainly on three aspects: (1) the strong vasodilatory effects of (endo)cannabinoids [58,59]; (2) the overactivation of endocannabinoid tone in hypertension [38,46], and (3) stronger hypotensive responses in hypertensive animals than in normotensive controls [46]. However, results regarding the beneficial effects of (endo)cannabinoids are based on in vitro experiments or in vivo ones after acute intravenous (*i.v.*) injection of compounds in anesthetized animals. Thus, the present review was aimed at determining (based on the available literature) the effects of chronic administration of (endo)cannabinoids on BP in various models of systemic and pulmonary hypertension. Moreover, we compared changes in the heart, arteries, kidneys, brain, blood, and lungs (if applicable) (i.e., organs/tissues important for the development of the above types of hypertension) and the liver to determine whether particular changes are tissue-dependent. We focused on changes in functional cardiac and vessel (mainly endothelial-dependent) responses, components of the endocannabinoid system, and markers of oxidative stress and inflammation since, according to the modified Dr. Page's Mosaic Theory of hypertension [8], hypertension is the result of many factors, including, among others, cardiac output [60], vascular reactivity (mainly endothelial-dependent) [61], oxidative stress [62], and inflammation [63], which interact to raise BP and cause end-organ damage.

6. Cannabinoids and the Endocannabinoid System

Cannabinoids are a group of compounds that were first isolated from *Cannabis sativa*. The most abundant plant-derived molecules from this group are Δ^9-tetrahydrocannabinol (THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), cannabichromene (CBC), Δ^9-tetrahydrocannabivarin (THCV), cannabivarin (CBV), and cannabidivarin (CBDV) [64]. It was not until the early 2000s that there was increased interest in other phytocannabinoids, including non-intoxicating CBD. Forty years of research into the mechanism of action of THC led to the discovery of cannabinoid receptors (CBRs), along with their endogenous ligands and metabolic enzymes, which together form the endocannabinoid system. Currently, we distinguish three main groups of cannabinoids: (1) the phytocannabinoids listed above; (2) synthetic cannabinoids, including WIN55212-2, CP55940, and JWH133; and (3) endocannabinoids (eCBs), which are produced endogenously and have an affinity to classical CBRs or endocannabinoid-like compounds. Despite their similar chemical structure to eCBs, the latter compounds hardly bind to classical CBRs but can interact with other elements of the endocannabinoid system. The best-known eCBs are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), whereas noladin ether (2-AGE), 2-linoleoylglycerol (2-LG), N-arachidonoyl-L-serine (ARA-S), dihomo-γ-linolenoyl ethanolamide (DGLEA), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), homo-γ-linolenyl ethanolamide (HEA), linolenoyl ethanolamide (LEA), N-arachidonoyl dopamine (NADA), N-arachidonoyl glycine (NAGly), oleamide, oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), palmitoleoyl ethanolamide (POEA), stearoyl ethanolamide (SEA), and virodhamine are endocannabinoid-like compounds [38]. Among them, PEA and OEA are gaining popularity in the scientific community because of their beneficial effects, such as anti-inflammatory, anti-anaphylactic, analgesic, and hypophagic activity, as well as maintenance of glucose homeostasis [65]. Moreover, for decades, PEA, a plant-derived dietary supplement or nutraceutical, has been considered to have immunomodulatory properties [66–68].

(Endo)cannabinoids act via two types of G protein-coupled receptors (GPCRs), cannabinoid receptor CB_1 (CB_1R) and CB_2 (CB_2R). CB_1Rs are spread all over the body but are mostly found in the central nervous system (CNS), which is the reason for the psychoactivity of THC. As shown in Figure 1, their activation exerts both pro-hypotensive and pro-hypertensive activity [39,45,46,69–71]. The hypotensive effects result mainly from a decrease in noradrenaline release from the sympathetic nerve endings innervating resistance vessels by the activation of presynaptic CB_1Rs and direct vasodilatory effects determined in various (but not all) vessels [46,58]. However, it should be remembered that CB_1Rs are also known for their pro-oxidant and pro-inflammatory effects, and their activation in the CNS leads mainly to a pressor response [39,45,46,71,72]. The highest density of CB_2Rs occurs in the immune system. In contrast to CB_1Rs, stimulation of CB_2Rs leads to anti-inflammatory and anti-oxidant influences and other antihypertensive effects [73].

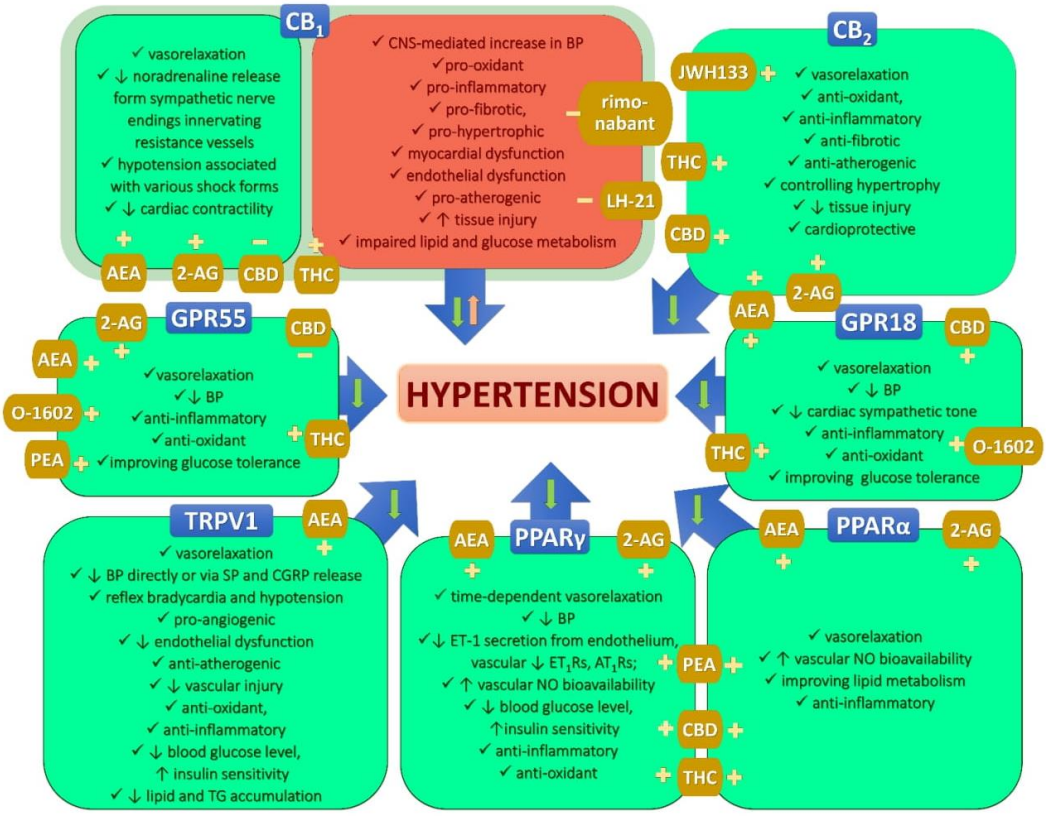

Figure 1. Well-known potential effects (not only related to the cardiovascular system) of compounds described in tables after interacting with classical and non-classical cannabinoid receptors. For receptor affinity, see [74–78]. For references regarding effects of particular receptors, see Section 6. Green indicates pro-hypotensive and red pro-hypertensive effects. Arrows next to effects indicate increase (↑) or decrease (↓); arrows in the center indicate predominantly pro-hypotensive (↓) or hypertensive (↑) effects. (+) activation, (−) blockade. 2-AG—2-arachidonoyl glycerol; AEA—anandamide; AT_1Rs—angiotensin II type 1 receptors; BP—blood pressure; CB_1—cannabinoid type 1 receptor; CB_2—cannabinoid type 2 receptor; CBD—cannabidiol; CGRP—calcitonin gene-related peptide; CNS—central nervous system; ET-1—endothelin 1; ET_1Rs—endothelin 1 receptors; NO—nitric oxide; PEA—palmitoyl ethanolamide; PPAR—peroxisome proliferator-activated receptor; SP—substance P; THC—Δ^9-tetrahydrocannabinol; TG—triglycerides; TRPV1—transient receptor potential vanilloid 1.

Apart from the classical ones, many different receptors may interact with both endo- and exogenous cannabinoids, such as orphan receptors GPR18 and GPR55, ionotropic transient receptor potential vanilloid type 1 (TRPV1), and peroxisome proliferator-activated receptors (PPARs) [79,80]. AEA is an endogenous ligand of TRPV1 receptors, the activation of which causes vasodilatation and other actions, leading to a decrease in BP (Figure 1) [69,81–83]. As shown in Figure 1, activation of GPR18 [84–86], GPR55 [77,87,88], PPARγ [75,89–92], or PPARα [89,91,92] can also lead to a drop in BP. Importantly, all of the above receptors are also present in the vascular and cardiac systems.

Despite slight variations by strain and vessel type, most cannabinoid receptors are expressed in both endothelium and smooth muscle cells of systemic vessels; however, sometimes, their expression/staining is more pronounced in endothelial cells [93–95]. The expression of GPR18 receptors in peripheral blood vessels is still a subject of debate [86]. CB_1Rs, CB_2Rs, TRPV1, GPR18, and GPR55 receptors are also expressed in pulmonary arteries (mostly evidenced in human studies), predominantly in the whole vessel wall,

although some papers show an increased presence of CB_1Rs in smooth muscle cells or, inversely, a prevalence of GPR18 receptors in the endothelium and adventitial layer of the vessel [48,93]. There are practically no studies comparing expression levels between systemic and pulmonary circulation, and most studies show a similar distribution of cannabinoid receptors throughout the vessels in both.

Cardiac CBRs are also widely distributed. CB_1Rs and CB_2Rs are present in the left ventricle, left and right atrium, and epicardial adipose tissue in humans and animals. GPR55 and GPR18 receptors were found in the left ventricle. Except for cardiac muscle tissue, CBRs are also present in coronary arteries but are absent from the electrical conduction system of the heart [78].

Due to the short biological half-life of eCBs, much attention is paid to their degradation process. Two main enzymes responsible for the catalysis of CBR ligands are fatty acid amide hydrolase (FAAH) (AEA and partially 2-AG) and monoacylglycerol lipase (MAGL) (mostly 2-AG). Their respective inhibitors, URB597 and JZL195, are used to enhance the endocannabinoid tone [38,80].

7. Vasodilatory Effects of Chosen (Endo)cannabinoids

As mentioned above, the strong vasodilatory effect of (endo)cannabinoids is one of the reasons they are suggested to possess potential anti-hypertensive and cardio- or vasculoprotective activity [58,59,69]. Table 2 presents the vasodilatory effects of all compounds examined in chronic experiments on hypertensive models (for descriptions, see Sections 9–11), which were examined in both normo- and hypertensive conditions in vitro. Indeed, as shown in Table 2, AEA (as well as its stable analog, methanandamide (MethAEA)), CBD, and THC exert direct vasodilatory effects. Importantly, their vasorelaxant action shows higher efficacy (up to 100% maximal effect) in resistance (mesenteric bed and small mesenteric arteries (sMAs)) [93,96–99], but much lower (up to 20%) in conductive systemic vessels (aorta, superior mesenteric arteries) [96,99,100]. One paper reported stronger relaxation of mesenteric arteries in response to AEA in female rats [97]; however, other experiments were performed on males.

The vasodilatory effects of (endo)cannabinoids (mainly their potency) depend on the hypertension model and vessel type (Table 2). Thus, the responses of resistance mesenteric arteries to AEA, MethAEA, and CBD were diminished in SHR [93,95,96] but enhanced in DOCA-salt [93,101] and unchanged in hypertension induced by chronic administration of L-NAME [98]. The only exception was the increase in potency but the decrease in the efficacy of the vasodilatory action of AEA in the mesenteric arteries of females [97]. In contrast, AEA showed stronger efficacy in the thoracic aorta of SHR [96] and rats with renal hypertension [100]. The vasodilatory effect of THC was enhanced in mesenteric arteries isolated from rats with hypertension induced by chronic L-NAME administration. Interestingly, small constriction and relaxation in the aorta in response to THC were noted in normotensive rats and rats with L-NAME-induced hypertension, respectively [99].

The most important mechanisms underlying the relaxant properties of (endo) cannabinoids are (1) stimulation of classical CBRs (CB_1 and/or CB_2), (2) stimulation of TRPV1 receptors, (3) activation of calcium channels, and (4) inhibition of calcium entry, along with (5) endothelium-dependent mechanisms (such as stimulation of hypothetical CB_X receptors) [59]. As shown in Table 2, a similar mechanistic approach can apply to hypertension. The most significant components of vascular response in this pathological condition are CBRs and endothelium. Interestingly, CB_1Rs mostly participate only in the hypertensive response [95,101]. Similar effects of AEA and MethAEA suggest that AEA does not act via its metabolites in mesenteric arteries (Table 2).

Table 2. Direct acute effects of (endo)cannabinoids on arteries isolated from rats (if not otherwise specified) with systemic or pulmonary hypertension.

Compound	Model	Artery	E_{max} (%) (in Parentheses Concentrations in μM for Which E_{max} Was Obtained)		pEC_{50}		Suggested Mechanism of Action in Hypertension	Ref.
			N	H	N	H		
AEA	WKY vs. SHR	perfused mesenteric bed	~100 [1] (10)	~100 [1] (10)	7.1	6.3 *	↓ NO-dependent relaxation; TRPV1-dependent	[96]
		G3 mesenteric	98 (3)	70 * (10)	6.5	6.8 *	sex-dependent (stronger in female); TRPV1- and endothelium-dependent	[97]
		thoracic aorta	13 (30)	48 * (30)	8.1	7.9	endothelium-dependent; CB_1R- and TRPV1-independent	[96]
	L-NAME-induced	perfused mesenteric bed	100 (10)	107 (10)	6.5	7.1 *	-	[98]
			~90 [1] (10)	~90 [1] (10)	6.3	6.4	↑ sensory nerve-mediated activity	[96]
		G3 mesenteric	~70 [1,2] (30)	~70 [1,2] (30)	5.7	5.6	-	[98]
		thoracic aorta	25 (30)	33 (30)	6.7	6.6	CB_1R-, TRPV1-, NO- and PG-independent	[96]
	2K1C	thoracic aorta	4 (30)	44 * (30)	-	5.2	CB_1R-, CB_2R-, NO- and endothelium-dependent	[100]
	hypoxia [3]	isolated perfused lung	-	↑ pulmonary arterial tone (10)	-	-	FAAH-dependent metabolites;sex-dependent (stronger in females)	[102]
		large pulmonary	-	no effect (10)	-	no effect	-	
MethAEA	DOCA-salt	G3 mesenteric	84 (30)	85 (30)	4.9	5.6 *	TRPV1-dependent in N and H;CB_1R-dependent in H only	[101]
		aorta	84 (30)	41 * (30)	6.1	n.d.	-	
	SHR	G3 mesenteric	97 (30)	98 (30)	6.1	5.6 *	CB_1R-dependent in H only	[95]
	hypoxia [3]	isolated perfused lung	-	no effect (10)	-	-	-	[102]

Table 2. *Cont.*

Compound	Model	Artery	E_{max} (%) (in Parentheses Concentrations in μM for Which E_{max} Was Obtained)		pEC_{50}		Suggested Mechanism of Action in Hypertension	Ref.
			N	H	N	H		
CBD	DOCA-salt	G3 mesenteric	92 (30)	91 (30)	5.5	5.9 *	CB_1R-, CB_2R- and endothelium-dependent	[93]
	SHR		93 (30)	82 (30)	6.0	5.6 *	CB_1R-dependent; CB_2R- and endothelium-independent	
	Hypertension [4]	pulmonary	94 (30)	93 (30)	4.9	4.1 *	endothelium, PG- and TRPV1-dependent;CB_1R-, CB_2R-independent	
THC	L-NAME-induced	G3 mesenteric	~60 [1] (100)	~70 [1] (100)	5.6	6.1 *	CB_1R-independent; ↑ sensory nerve-mediated activity and PG-dependent	[99]
		G0 mesenteric	16 (100)	38 * (100)	-	-	-	
		aorta	5—constriction (100)	4—relaxation (100)	-	-	-	

[1] No precise data given, calculated from the figures in the publication. [2] Maximal effect was not determined. [3] Mouse model. [4] Human studies. * Significant difference at a level of at least $p < 0.05$ compared to normotension. n.d., not determined because of the too-low value of E_{max}. ↑ increase; ↓ decrease; 2K1C—Goldblatt two-kidney, one-clip model; AEA—anandamide; CB_1R—cannabinoid receptor type 1; CB_2R—cannabinoid receptor type 2; CBD—cannabidiol; DOCA—deoxycorticosterone acetate; E_{max}—maximal effect; FAAH—fatty acid amide hydrolase; G0—superior mesenteric artery (conduit); G3—third-order branches mesenteric artery (resistance); H—hypertension; L-NAME—L-N^G-nitro arginine methyl ester; MethAEA—methanandamide; N—normotension; NO—nitric oxide; pEC_{50}—the negative logarithm of the half maximal effective concentration; PG—prostanoids; Ref.—references; SHR—spontaneously hypertensive rat; THC—Δ^9-tetrahydrocannabinol; TRPV1—transient receptor potential vanilloid 1; WKY—Wistar-Kyoto rat.

In addition to AEA, other eCBs and endocannabinoid-like compounds possess vasodilatory potencies, such as 2-AG, 2-AGE, ARA-S, NADA, NAGly, OEA, PEA, oleamide, and virodhamine [48,58]. However, they were not examined under hypertensive conditions. Sometimes they do not act directly but through their anti-inflammatory and vasodilatory ω-3 eCB epoxide regioisomer metabolites [103]. In addition, endocannabinoid-like compounds (e.g., OEA and PEA) [104] can also intensify the action of eCBs by competing with them for metabolizing enzymes, thus reducing their degradation (the so-called entourage effect) [38]. Interestingly, 2-AG induced contraction of rat aorta via vasoconstrictor metabolites [105]. The vascular activity of other eCBs and endocannabinoid-like compounds has not yet been examined.

8. Acute In Vivo Cardiovascular Effects of (Endo)cannabinoids

We previously reviewed the cardiovascular effects of (endo)cannabinoids in normotension [69] and systemic hypertension [46]. Briefly, the effects of eCBs on BP and heart rate (HR) are complex and vary depending on whether the animal is anesthetized or not [69]. In rats anesthetized with urethane, intravenous (*i.v.*) injection of AEA and its stable analog MethAEA resulted in a three-phase cardiovascular response. Phase I is characterized by rapid and marked bradycardia and a transient drop in BP (the so-called Bezold–Jarisch reflex), resulting from the activation of TRPV1 receptors located on cardiac afferents of the vagus fibers. It is not determined after acute *i.v.* administration of THC, CBD, or synthetic cannabinoids that do not activate TRPV1 receptors. Phase II (also observed after injection of MethAEA and THC) consists of a short-term pressure response (lasting approx. 30–60 s) associated with increased contractility of the heart and blood flow through the kidney and mesenteric bed. It results mainly from stimulation of the brain's CB_1Rs, glutamatergic NMDA, thromboxane A_2 (TP), and β_2-adrenergic receptors [69]. In phase III (also observed after injection of MethAEA, THC, and synthetic cannabinoids), there is a prolonged (up to 10 min) significant drop in BP, accompanied by decreased renal and mesenteric flow, a significant reduction in myocardial contractility, and a slight decrease in HR and vascular resistance. Phase III is suggested to result from [69]: (1) stimulation of presynaptic CB_1Rs located at the ends of sympathetic fibers innervating blood vessels and the heart, inhibiting the release of norepinephrine; (2) stimulation of hypothetical CB_X endothelial vasodilating receptors; and (3) the CB_1R-mediated negative inotropic effect of (endo)cannabinoids in the heart.

In conscious animals, the predominant effect of AEA, THC, and synthetic cannabinoid administration is the pressure response combined with the narrowing of the renal blood vessels and the mesentery. This mainly results from central activity [69]. Interestingly, an increase in arterial pressure, plasma noradrenaline concentration, and renal sympathetic tone has been observed after intracerebroventricular (*i.c.v.*) administration of synthetic cannabinoids or AEA in both anesthetized and conscious animals [69]. Similarly, stimulation of CB_1Rs in the paraventricular nucleus of the hypothalamus (PVN) causes a pressor response in both anesthetized and conscious rats, clearly suggesting that central mechanisms are responsible for the increased BP induced by cannabinoids [71].

Unlike AEA, 2-AG caused only a monophasic response in the circulatory system of rats and pentobarbital- and/or urethane-anesthetized mice with hypotension and tachycardia, lasting about 10–18 min. However, the pressure drop observed does not depend on 2-AG itself, but on the arachidonic acid metabolites formed from 2-AG [69].

The endogenous endocannabinoid tone is not involved in regulating the cardiovascular system under physiological conditions since none of the CBR antagonists, inhibitors of eCBs metabolism, or genetic deletions of components of the endocannabinoid system modify cardiovascular parameters [69]. The situation is different under pathophysiological conditions [46]. For example, (1) acute *i.v.* injection of AEA and MethAEA induced stronger hypotension in anesthetized SHR as well as different models of secondary hypertension than in respective normotensive controls; and (2) two CB_1R antagonists, rimonabant and AM251, further increased and two FAAH inhibitors, URB597 and AM3506, decreased

the elevated BP and cardiac contractility in hypertensive animals and did not affect any hemodynamic parameters in normotensive controls.

Such promising results demonstrate the strong vasodilatory effects of (endo)cannabinoids in isolated resistance arteries (see Section 7) and the involvement of the endocannabinoid tone in cardiovascular system regulation in hypertension, and the more evident hypotensive response to these compounds in hypertension (see above) suggests potential beneficial therapeutic effects. Experiments with the chronic administration of (endo)cannabinoids allowed for verification of the above theory.

9. Cardiovascular Effects of Chronic (Endo)cannabinoid Administration in Hypertension

Table 3 shows the results from all publications regarding the influence of chronic administration of (endo)cannabinoids or compounds modifying the endocannabinoid tone on BP and HR in experimental models of hypertension and a few cases in human trials. Particular compounds were studied in both hypertensive and normotensive control groups. Importantly, the compounds did not significantly affect BP in normotensive individuals. The amplitude of changes in BP (both decreases and increases) depended on their basal values. The lack of changes in normotension can be explained by too low basal pressure. However, in experiments performed on isolated vessels (see Table 2 and Section 7), (endo)cannabinoids elicited full or almost full vasorelaxation of pre-constricted resistance arteries isolated from normotensive and hypertensive donors. Interestingly, cannabinoids affected HR in hypertension in only two cases [87,106], which indicates that different mechanisms are involved in the regulation of BP and HR. It should be remembered that the main effect of marihuana in humans is tachycardia, in contrast to the bradycardia noticed in animals after acute (endo)cannabinoid injection [76,78].

The first group of cannabinoids studied in hypertension was exogenously administrated eCBs or compounds inhibiting their metabolism. As shown in Table 3, only one studied endocannabinoid-like compound, PEA, confirmed the working hypothesis that a compound exerting strong vasodilatory activity [104] could also possess hypotensive potential after chronic application. Indeed, after 5 weeks of subcutaneous (*s.c.*) PEA administration in SHR rats [107,108], a strong hypotensive effect was noticed. The lack of such action before then (weeks 1–4) might have resulted not only from the vasodilatation but also from the protection against kidney injury (for details, see Section 10.4).

In contrast to distinct and prolonged hypotension observed after acute injection with the main eCB, AEA, or the inhibitor of its degradation, URB597, in hypertensive rats (see Section 8), such a promising effect was not noted after chronic administration (see Table 3). Thus, AEA tended to increase BP in Dahl salt-sensitive rats (with a high-salt diet) [109], while it decreased BP in SHR [110,111]. This discrepancy in the effects probably does not result from small differences in doses or procedure duration (3 vs. 5 mg/kg and 2 vs. 4 weeks, respectively) but from the form, route, and frequency of administration. Golosova et al. [109] experimented with *i.v.* AEA administration in its unmodified form once daily, whereas Martín Giménez et al. [110,111] used a nanoformulated compound and gave it intraperitoneally (*i.p.*) once weekly. Unaltered compounds with 100% bioavailability and no first-pass effect acted more strongly and aggressively, but for a shorter time because of their rapid metabolism. The nanoformulated version was released slowly, and the action was more delayed. Kidney injury has been suggested as the direct cause of the hypertensive effect of *i.v.* AEA (see Section 10.4), which might be induced by repeated administration of toxic concentrations of the compound. It is possible that a cardiotoxic effect of AEA described previously in vitro [112] could also occur in this model.

Table 3. Cardiovascular effects of chronic administration of (endo)cannabinoids in different models of systemic hypertension in male rats (unless noted otherwise).

Compound, Dose, and Protocol	Model	BP and HR Effects	Influence on Changes Induced by Hypertension		References
			Cardiac Effects/Expression in Heart (If Not Stated Otherwise)	Vascular Effects	
PEA 30 mg/kg, *s.c.*, once daily, 5 weeks	SHR	- ↓SBP (only in the 5th week of the treatment; by ~50–60 mmHg) - ↔HR	n.d.	vasodilatory effects in mesenteric or carotid arteries: - ↑EDHF-mediated relaxation to Ach; - ↑synthesis/release of vasodilatory EETs, NO, and PGI_2 and/or ↓EETs degradation; - ↓RAAS activity (↓ACE and AT_1R signaling pathway); anti-inflammatory effects: ↓NF-κB signaling pathway	[107,108]
AEA 3 mg/kg, *i.v.*, once daily, 14 days	Dahl salt-sensitive + high salt (8%) diet	- consistent trend to ↑MBP at the 2nd week of the treatment (by ~20 mmHg)	n.d.	n.d.	[109]
nf-AEA 5 mg/kg, *i.p.*, once weekly, 4 weeks	SHR	- ↓SBP after 4 weeks (by 35 mmHg) [1]	anti-hypertrophic effects: ↓ventricular mass and LV hypertrophy indexes	n.d.	[110,111]
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	DOCA-salt	- ↓SBP (after 2 weeks by ~30–60 mmHg) - ↔ HR	anti-hypertrophic effects: - ↓cardiac (only in younger) and LV hypertrophy - ↓medium and large coronary artery thickness in LV cardiac functional effects: - ↓diastolic stiffness - tendency to ↑cardiostimulatory effects of isoprenaline: contractility, cardiac work and inotropism - normalization of (-) inotropic effect of CB_1R agonism anti-oxidant effects: ↓ROS, 4-HNE, CO gr., XO, NADPH oxidase activity and ↑GSH, GSH/GSSG, vit. C, ↑Nrf2, p21, ↓Keap1 pro-oxidant effects: ↓GSH-Px, GSSG-R, Cu-Zn-SOD, Trx-R activity and ↑MDA, 8-OHdG, ↓Trx, vit. A, ERK1/2, HO-1, MAPK pro-inflammatory effects: ↑TNFα endocannabinoid effects: - ↑FAAH in LV, ↓FAAH, MAGL activity - tendency to ↓CB_1R and CB_2R in LV but ↑CB_1R and CB_2R in whole heart - ↑TRPV1, GPR55, PPARα, ↓PPARγ - ↑NADA and 2-AG other effects: - ↑heart availability of energy substrates - ↑intramyocardial glycogen storage - ↓apoptosis (↓ Bax, caspase 3, 9)	vasodilatory effects: ↓response to phenylephrine in sMAs anti-hypertrophic effects: ↓medial thoracic aorta hypertrophy endocannabinoid effects: ↓FAAH in sMAs other effects: ↑$K_{Ca}3.1$ sMAs	[101,113–120]

Table 3. *Cont.*

Compound, Dose, and Protocol	Model	BP and HR Effects	Influence on Changes Induced by Hypertension		References
			Cardiac Effects/Expression in Heart (If Not Stated Otherwise)	Vascular Effects	
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	SHR	- ↔SBP or slight ↓SBP (by ~20 mmHg after 2 weeks) and HR	hypertrophic effects: ↑heart hypertrophy but ↓LV hypertrophy cardiac functional effects: - ↑(+) chronotropic effect of isoprenaline - normalization of (+) inotropic effect of isoprenaline in atria anti-oxidant effects: ↓XO, ↑CAT, Trx-R activity, ↑GSH, GSH/GSSG, vit. E, C, Trx, ↓Keap1, Bach1, ↑ERK1/2, MAPK pro-oxidant effects: ↓GSH-Px activity and ↑MDA, 4-HNE, 8-isoprostanes, 8-OHdG, CO gr., ↓Nrf2, Keap1, HO-1 anti-inflammatory effects: ↓TNFα endocannabinoid effects: ↑CB_1R, CB_2R, GPR55, PPARγ and ↓TRPV1, PPARα - translocation of CB_1R immunoreactivity to the intercalated discs in LV - tendency to ↑FAAH in LV - ↓FAAH and MAGL activity - ↑AEA, NADA, and 2-AG other effects: - ↑cardioprotective LV sphingolipid (S1P) - ↑palmitate uptake by LV cardiomyocytes - protection from DAG and CER accumulation in LV - improvement of insulin signaling in LV - ↓free AA - ↓apoptosis (↑Bcl-2, ↓Bax, caspase 3, 8, 9)	vasodilatory effects - ↓phenylephrine-mediated CB_1R-independent vasoconstriction in sMAs - ↑potency of Ach-mediated endothelium-dependent vasorelaxation in sMAs and aorta - ↑potency of MethAEA-mediated CB_1R-independent vasorelaxation vasoconstrictive effects: ↑vasoconstrictive potency of U46619 (thromboxane analog) in sMAs anti-hypertrophic effects: tendency to ↓sMAs wall hypertrophy endocannabinoid effects - ↑2-AG in aorta, ↑AEA in sMAs and aorta - ↓CB_1R in aorta	[95,116,117,120–122]
JZL195 10 mg/kg, *i.p.*, once daily, 14 days	SHR	- tendency to ↓BP (by ~20 mmHg after 2 weeks) - ↔HR	- no changes in cardiac hypertrophy	n.d.	[123]
rimonabant 20 mg, oral, once daily, 12 months	hypertension [2]	- ↓SBP by ~13 and 7 mmHg and DBP by ~6 and 2 mmHg in H. and N. patients, respectively	n.d.	n.d.	[124]
rimonabant 20 mg, oral, once daily, 12 months	hypertension [2]	- ↓SBP by ~3 and 0.5 mmHg and DBP by ~2 and 0.5 mmHg in H. and N. patients, respectively	- reductions more evident in patients with higher cardiometabolic risk (e.g., dyslipidemia and type 2 diabetes) - the hypotensive effect seems to be mediated by weight loss		[125]
rimonabant 20 mg, oral, once daily, 24 months	hypertension [2]	- ↓SBP by ~1.5 and 0.5 mmHg and DBP by ~2 and 0.5 mmHg in H. and N. patients, respectively	- changes not significantly different from placebo		[126]

Table 3. *Cont.*

Compound, Dose, and Protocol	Model	BP and HR Effects	Influence on Changes Induced by Hypertension		References
			Cardiac Effects/Expression in Heart (If Not Stated Otherwise)	Vascular Effects	
rimonabant 10 mg/kg, oral, once daily, 3 weeks	(mRen2)27 higher RAAS activity	- ↓SBP (by ~25 mmHg within 24 h and remained lower through 3 weeks); ↔HR - better sympathetic and parasympathetic baroreflex sensitivity	n.d.	n.d.	[127]
LH-21 1 mg/kg, 3 mg/kg, *i.p.*, 3 weeks	KKAγ mice (BP was ↑ by about 10 mmHg only) [3]	- normalization of SBP, DBP, MBP (only for 3 mg/kg) - ↔HR	n.d.	anti-inflammatory effects on aorta: - ↓ICAM-1, MCP-1, TNFα mRNA - ↓lipocalin-2	[128]
JWH133 1 mmol/l, 10 µL, *i.c.v.*, once daily, 4 weeks	SHR (conscious and anesthetized)	- ↓MBP and HR by ~35 mmHg and 70 beats/min respectively after 2 weeks of administration	n.d.	n.d.	[106]
O-1602 0.25 mg/kg, *i.a.*, once daily, 14 days	SHR [3]	- ↓MBP by ~30 mmHg [1] - ↑HR by ~50 beats/min [1]	n.d.	other effects: ↓RhoA/Rho-kinase signaling in aorta	[87]
CBD 10 mg/kg, *i.p.*, once daily, 14 days	DOCA-salt	- ↔HR, SBP, DBP, and MBP	anti-hypertrophic effects: ↓width of LV cardiomyocytes cardiac functional effects - ↓carbachol-induced vasoconstriction of coronary arteries - ↑(-) inotropic effect of CB_1R agonism - ↑lusitropic effects: (+) isoprenaline and (-) carbachol anti-oxidant effects: ↓MDA, ↓GSSG, ↑GSH and small ↓4-HNE pro-oxidant effects: small ↓vit. A and E endocannabinoid effects: - ↓2-AG, OEA, DEA, DGLEA - ↓FAAH activity - small ↓CB_1R, CB_2R, and GPR18 other effects: - ↑FFA LA and ↓ FFA AA - ↓β_1-adrenoceptor in LV	vasodilatory effects: - ↑Ach-induced endothelium-dependent vasorelaxation in aortas (NO-dependent) and sMAs - ↑eNOS in aortas and sMAs, ↑*NOS3* in sMAs, ↑*PGIS* in sMAs anti-hypertrophic effects: ↓aorta and sMAs hypertrophy endocannabinoid effects: - ↓CB_1R in sMAs but ↑*Cnr1* in aortas - ↑*Cnr2* in aortas and sMAs - ↑AEA, 2-AG, PEA, and DEA; tendency to ↑OEA, HEA, POEA, LEA, and 2-LG; ↓EPEA, DHEA, and NAGly in aorta other effects: - ↓vWF in aortas and sMAs - ↑*KCNN4* in aortas and sMAs - ↑*KCNN3* in sMAs	[94,129,130]

Table 3. *Cont.*

Compound, Dose, and Protocol	Model	BP and HR Effects	Influence on Changes Induced by Hypertension		References
			Cardiac Effects/Expression in Heart (If Not Stated Otherwise)	Vascular Effects	
CBD 10 mg/kg, *i.p.*, once daily, 14 days	SHR	- ↔HR, SBP, DBP, and MBP	anti-hypertrophic effects: ↓width of LV and RV myocytes and ↓RV hypertrophy cardiac functional effects - small ↓diastolic stiffness - ↓carbachol-induced vasoconstriction of coronary arteries - ↓(-) inotropic effect of CB_1R agonism - ↑lusitropic effects: (+) isoprenaline and (-) carbachol anti-oxidant effects: ↓4-HHE and tendency to ↓4-HNE, ↑GSH, and ↓GSSG pro-oxidant effects: ↓vit. A and E endocannabinoid effects: - small ↓FAAH activity - ↓GPR55 and small ↓CB_1R and GPR18 other effects: ↑FFA LA, FFA AA	vasodilatory effects: - ↑Ach-induced endothelium-dependent vasorelaxation in aortas and sMAs (COX dependent) - ↑eNOS in aortas and sMAs, ↑*NOS3* in aortas and sMAs, ↑*PGIS* in sMAs vasoconstrictive effects: ↓potency of SNP-induced vasorelaxation in sMAs anti-hypertrophic effects: ↓aorta and sMAs hypertrophy pro-inflammatory effects: ↑COX-1 in aorta endocannabinoid effects: - ↑CB_1R in sMAs and tendency to ↑*Cnr1* in aortas and sMAs - ↑*Cnr2* in aortas and sMAs - ↑TRPV1 in aortas - ↓AEA and small ↓2-AG, PEA, HEA, DEA, EPEA, DHEA, LEA, 2-LG, and NAGly in aortas other effects: - ↓vWF in aortas and sMAs - ↑*KCNN4* in aortas and sMAs - ↑*KCNN3* in sMAs	[94,129,130]
CBD 200 mg/kg, oral, 4 weeks	OLETF rats with metabolic syndrome	- ↔BP [1]	- loss of visceral adiposity was not associated with reduced BP		[131]
Δ^8-THC 3 mg/kg, *i.p.*, once daily, 14 days	ARH unilaterally adrenalectomized +1% NaCl [3]	- ↓BP (by ~13 and 15 mmHg at the end of the 1st and 2nd week)	n.d.	n.d.	[132]
Δ^9-THC 3 mg/kg, *i.p.*, once daily, 7 or 14 days		- ↓BP (by ~18 and 13 mmHg at the end of the 1st and 2nd week); - tolerance to the acute hypotensive effect of the compound (in a shorter protocol)	n.d.	n.d.	

Table 3. *Cont.*

Compound, Dose, and Protocol	Model	BP and HR Effects	Influence on Changes Induced by Hypertension		References
			Cardiac Effects/Expression in Heart (If Not Stated Otherwise)	Vascular Effects	
Δ^9-THC 1 mg/kg 2 mg/kg, *s.c.*, once daily, 3–5 weeks	metacorticoid and renal hypertension	- ↔BP and HR	n.d.	n.d.	[133]
Δ^9-THC 5–25 mg/kg (increasing dosing), oral, once daily, 5 or 10 days	SHR	- transient ↓BP after increasing the dose (tolerance developed) - ↓SBP after highest dose chronic treatment (with no tolerance effect)	n.d.	n.d.	[134,135]

The Table summarizes all significant effects described in particular publications. Non-significant results are not mentioned. [1] BP and HR were determined at endpoint only. [2] In humans, SBP was less than 165 mmHg and DBP less than 105 mmHg. [3] Female animals. ↑ increase; ↓ decrease; ↔ no effect; *i.a.*—intraarterial; *i.c.v.*—intracerebroventricular; *i.g.*—intragastrical; *i.p.*—intraperitoneal; *i.v.*—intravenous; *s.c.*—subcutaneous; 2-AG—arachidonoylglycerol; 2-LG—2-linoleoylglycerol; 4-HHE—hydroxyhexenal; 4-HNE—4-hydroxynonenal; 8-OHdG—8-hydroxy-2′-deoxyguanosine; AA—arachidonic acid; ACE—angiotensin-converting enzyme; Ach—acetylcholine; AEA—anandamide; ARH—adrenal regeneration hypertension; AT1R—angiotensin II type 1 receptor; Bach1—BTB and CNC homology 1 transcription factor; Bax—pro-apoptotic bcl-2-like protein 4; Bcl-2—B-cell lymphoma 2; BP—blood pressure; CAT—catalase; CB_1R—cannabinoid receptor type 1; CB_2R—cannabinoid receptor type 2; CBD—cannabidiol; CER—ceramide; *Cnr1*—gene encoding CB_1R protein; *Cnr2*—gene encoding CB_2R protein; CNS—central nervous system; CO gr.—protein carbonyl groups; COX—cyclooxygenase; Cu-Zn-SOD—cytosolic superoxide dismutase; DAG—diacylglycerol; DBP—diastolic blood pressure; DEA—docosatetraenoyl ethanolamide; DGLEA—dihomo-γ-linolenoyl ethanolamide; DHEA—docosahexaenoyl ethanolamide; DOCA—deoxycorticosterone acetate; EDHF—endothelium-derived hyperpolarizing factor; EETs—epoxyeicosatrienoic acids; eNOS—endothelial nitric oxide synthase; EPEA—eicosapentaenoyl ethanolamide; ERK—extracellular signal-regulated kinases; FAAH—fatty acid amide hydrolase; FFA—free fatty acids; GPR—G protein-coupled receptor; GSH—glutathione; GSH-Px—glutathione peroxidase; GSSG—glutathione disulfide; GSSG-R—glutathione reductase; H—hypertensive; HEA—homo-γ-linolenyl ethanolamide; HO-1—heme oxygenase 1; HR—heart rate; ICAM-1—intercellular adhesion molecule 1; *KCNN3*—gene encoding $K_{Ca}2.3$ protein; *KCNN4*—gene encoding $K_{Ca}3.1$ protein; Keap1—kelch-like ECH-associated protein 1; KKAγ mice, spontaneously diabetic; LA—linoleic acid; LEA—linolenoyl ethanolamide; LV—left ventricle; MAGL—monoacylglycerol lipase; MAPK—mitogen-activated protein kinase; MBP—mean blood pressure; MCP-1—monocyte chemoattractant protein-1; MDA—malondialdehyde; MethAEA—methanandamide; N—normotensive; NADA—N-arachidonoyl dopamine; NADPH—nicotinamide adenine dinucleotide phosphate; NAGLy—N-arachidonoyl glycine; n.d.—not determined; NF-κB—nuclear factor kappa-light-chain-enhancer of activated B cells; nf-AEA—nanoformulated anandamde; NO—nitric oxide; *NOS3*—gene encoding eNOS; Nrf2—nuclear factor erythroid 2-related factor 2; OEA—oleoyl ethanolamide; OLETF—Otsuka Long-Evans Tokushima Fatty type 2 diabetic rats; p21—cyclin-dependent kinase inhibitor 1; PEA—palmitoyl ethanolamide; PGI_2—prostacyclin; *PGIS*—gene encoding prostacyclin synthase; POEA—palmitoleoyl ethanolamide; PPAR—peroxisome proliferator-activated receptors; RAAS—renin-angiotensin-aldosterone system; ROS—reactive oxygen species; RV—right ventricle; S1P—sphingosine-1-phosphate; SBP—systolic blood pressure; SHR—spontaneously hypertensive rat; sMAs—small mesenteric arteries (resistance); SNP—sodium nitroprusside; THC—tetrahydrocannabinol; TNFα—tumor necrosis factor α; TRPV1—transient receptor potential vanilloid 1; Trx—thioredoxin; Trx-R—thioredoxin reductase; vit.—vitamin; vWF—von Willebrand factor; XO—xanthine oxidase.

Chronic administration of the FAAH inhibitor URB597, which mainly degrades AEA, modified BP in a model-dependent manner. In secondary DOCA-salt hypertension, it decreased BP after 2 weeks of treatment [113–116,119], whereas in SHR (primary hypertension), there was no change [116,117] or only a slight decrease [121]. This was probably due to the more dynamic development of hypertension in DOCA-salt vs. SHR (4 weeks vs. 8–10 weeks to obtain similar BP values). An alternative explanation has to do with model-dependent vasodilatory effects of (endo)cannabinoids in isolated vessels. As shown in Table 2, both MethAEA and CBD caused mesenteric vasodilatation, which was more potent in DOCA-salt hypertensive than in control animals, whereas in SHR, these effects were weaker than in normotension. Another inhibitor, JZL195, which inhibits both FAAH and MAGL and stops AEA and 2-AG degradation, only showed a tendency to lower BP in SHR [123]. This suggests that 2-AG does not intensify the hypotensive effect of AEA observed after URB597 administration.

Activation of CB_1Rs might increase BP via central effects or decrease BP via direct vasodilatation, reduce noradrenaline release from sympathetic nerve endings innervating resistance vessels, or decrease cardiac contractility [45,69]. The direct synthetic CB_1R antagonist rimonabant, acting nonspecifically on both the peripheral and central level, was also investigated as a potential antihypertensive agent. It was examined in a big clinical trial, Rimonabant in Obesity (RIO), mostly including obese, diabetic, or dyslipidemia patients. The results obtained for the extracted hypertensive group showed that one-year [124,125] or two-year [126] treatment with rimonabant resulted in only small decreases compared to normotension. However, it should be noted that only patients with BP below 165 mmHg were enrolled in the trial. Moreover, the hypotensive effect could be caused by weight loss.

In an animal model of (mRen2), 27 rats (a monogenetic model of Ang II-dependent hypertension in which the mouse renin *Ren2* gene is transfected into the Sprague–Dawley rat genome), a higher dose of rimonabant (10 mg/kg vs. 20 mg in a clinical trial) caused a significant pressure drop [127]. Except for the difference in dose, in the animal experiment, there was also higher basal pressure. Importantly, the hypotensive effect appeared as early as 24 h after CB_1R antagonist administration and remained lower for 3 weeks of examination. Interestingly, acute *i.v.* rimonabant injection increased BP in SHR [136] but decreased it in (mRen2)27 rats [127], again proving that the potential hypotensive effects of (endo)cannabinoids are model-dependent. Isolated peripheral blockade of CB_1Rs by LH-21 normalized slightly increased BP in spontaneous diabetic KKAγ mice [128]. Thus, the beneficial effect of antagonizing CB_1Rs also has a peripheral component. However, the fact that antagonists of CB_1Rs were effective in hypertension contradicts the use of compounds that stimulate these receptors in this indication, including the aforementioned eCBs and/or compounds that increase their concentration. What is more, antagonists of CBRs caused an effect that was more explicit and intense. Unfortunately, the compounds stimulating CBR and CB_1R antagonists were examined in different models of hypertension.

Other single targets studied in hypertensive animals (SHR in both cases) were CB_2R and GPR55 receptors. 28-day-lasting *i.c.v.* administration of CB_2R agonist JWH133 resulted in a distinct fall in BP [106]. A similar reduction in BP occurred when O-1602, a GPR55 receptor (and to a lesser extent GRP18) agonist was used intra-arterially (*i.a.*) for 2 weeks [87]. Interestingly, the influence of chronic administration of compounds affecting the endocannabinoid system on HR was noted only in these two cases (JWH133 decreased it, and O-1602 increased it in hypertensive animals).

As shown in Table 3, the potential hypotensive influence of chronic administration of two phytocannabinoids was also examined in experimental hypertension. The first one, CBD at a dose of 10 mg/kg administered over 2 weeks, failed to diminish BP in both DOCA-salt and SHR [129]. Even a much higher dose of CBD (200 mg/kg) did not improve BP-related effects in OLETF rats with mild obesity, the clinical onset of diabetes mellitus, and metabolic syndrome [131]. Better effects were found with Δ^8- and Δ^9-THC; however, there is variability among performed studies. Low *s.c.* dose (1 mg/kg) of Δ^9-THC did not alter BP in metacorticoid or renal hypertension [133]. A higher dose (3 mg/kg) given *i.p.*

was effective in ARH for both Δ^8- and Δ^9-THC, although a longer scheme (14 days) did not lead to tolerance induction [132]. The highest doses of Δ^9-THC (5–25 mg/kg), administered orally, resulted in a stable decrease in BP after the highest dose [135] and transient lowering of pressure after increasing the lower dose in SHR, after which tolerance was induced [134].

We could not determine whether the effects induced by chronic (endo)cannabinoid administration are gender-dependent since most of the experiments were performed on male animals, and none of the compounds have been studied under comparable conditions in both sexes (see Tables 2 and 3).

The choice of route of administration in the described studies should not be surprising. The authors mostly used *i.p.* and *s.c.* injections, and in only a few cases (mostly in clinical trials) oral administration. These are the easiest to perform and give the full dose of the administered compound, although they are unlikely to be translated into clinical trials and further into clinical practice. So, if a compound shows promising effects, it should be tested using a more approachable route of administration: oral or inhalation. The latter is especially interesting since it is the most common route for recreational cannabis use and also for many cannabinoid-based drugs [137,138]. To date, no studies on chronic hypertension with inhaled (endo)cannabinoids have been performed. However, we would like to point out that THC increases HR in humans independent of its route of administration (including inhalation, oral, or even *i.v.*) [78], so the effects of the examined (endo)cannabinoids may also stay the same regardless of their formulation.

Unfortunately, so far, there is no publication regarding the influence of chronic cannabis use, either recreationally or therapeutically, in patients with hypertension. We can only suppose that, similar to the results obtained using experimental hypertension models, their final effect on BP would depend on whether they stimulate one or more targets. Moreover, it should be kept in mind that (1) there are species differences (e.g., acute administration of THC causes tachycardia in humans and bradycardia in experimental animals) [78], and (2) marijuana and synthetic cannabimimetics can induce acute myocardial infarction (MI) in healthy young people [78]. For example, a recent analysis of the UK Biobank dataset demonstrated that cannabis use was a statistically significant positive predictor for MI [139].

10. Potential Mechanisms of Cardiovascular Effects of Chronic (Endo)cannabinoid Administration in Hypertension

As shown in Tables 3 and 4 and Figure 2 and listed below, several potential mechanisms of antihypertensive effects were investigated in the examination of cardiovascular effects of chronic (endo)cannabinoid administration in various hypertension models. The tables summarize only significant effects described in particular publications; non-significant results are not mentioned. In the description below and Figure 2, we include only the most important mechanisms listed in the modified Dr. Page's Mosaic Theory of hypertension [8] (see Section 5) and the most intensively studied after chronic (endo)cannabinoid administration.

Table 4. Effects of chronic administration of (endo)cannabinoids in various tissues of different models of systemic hypertension in male rats (unless noted otherwise).

Compound, Dose, and Protocol	Model	Effects	References
		CENTRAL NERVOUS SYSTEM	
nf-AEA 5 mg/kg, *i.p.*, once weekly, 4 weeks	SHR	anti-inflammatory/-oxidant effects: ↓WT-1, AT_1R, iNOS, and ↑Hsp70 in brain cortex other effects: ↓apoptosis (TUNEL and caspase-3) in brain cortex	[111]
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	SHR	anti-oxidant effects in brain: - ↑Cu-Zn-SOD, GSH-Px, GSSG-R activity, ↓MDA, ↑vit. E - ↑Nrf2 and HO-1 and ↓Bach1 endocannabinoid effects in brain: - ↓FAAH activity and ↑AEA - ↓CB_2R and ↑GPR55 other effects: ↓phospholipid but ↑free AA, DHA, and LA in brain	[140]
JWH133 1 mmol/l, 10 µL, *i.c.v.*, once daily, 4 weeks	SHR	anti-inflammatory effects: ↓IL-1β, IL-6, and TNFα in RVLM	[106]
		BLOOD	
nf-AEA 5 mg/kg, *i.p.*, once weekly, 4 weeks	SHR	anti-inflammatory effects: ↓IL-1, IL-6, TNFα, uCRP, and Hsp70 in serum anti-oxidant effects: ↓NADPH oxidase serum activity and ↑nitrites (an indirect measure of NO) in serum	[111]
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	DOCA-salt	anti-oxidant effects: ↑GSH, ↓MDA in plasma, and ↓MDA in erythrocytes pro-oxidant effects: ↓ plasma GSH-Px activity endocannabinoid effects: - ↑AEA and NADA but ↓2-AG in plasma - ↓CB_1R, CB_2R, TRPV1, GPR55 in lymphocytes other effects: - ↑plasma insulin and ↑insulin sensitivity (HOMA-IR, QUICKI, and FGIR) - ↑anti-aggregation effect (↑sialic acid in erythrocytes, sialic acid in plasma and ↑negative charge of the erythrocyte membrane) - normalization of electrochemical properties of erythrocyte; ↓erythrocyte size - ↓phospholipid AA and ↑free AA, DHA, LA in plasma - ↑phospholipids in erythrocytes membrane (PC, PS, and PE)	[115,117,118,141]

Table 4. *Cont.*

Compound, Dose, and Protocol	Model	Effects	References
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	SHR	anti-oxidant effects: ↑GSSG-R plasma activity and ↓MDA in erythrocytes pro-oxidant effects: ↑plasma ROS, MDA, and ↓GSH in erythrocytes endocannabinoid effects: - ↑AEA, NADA, and 2-AG in plasma - ↑TRPV1 and ↓CB_2R in lymphocytes other effects: - ↓plasma insulin and ↓ insulin sensitivity (HOMA-IR) - ↑anti-aggregation effect (↑sialic acid in erythrocytes, ↓sialic acid in plasma and ↑negative charge of the erythrocyte membrane) - normalization of electrochemical properties of erythrocyte, ↓erythrocyte size - ↓phospholipid DHA in plasma, ↑phospholipids in erythrocytes membrane (PC, PS, PE, and PI)	[117,122,141]
rimonabant 10 mg/kg, oral, once daily, 4 weeks	(mRen2)27	other effects: ↓serum leptin and insulin	[127]
CBD 10 mg/kg, *i.p.*, once daily, 14 days	DOCA-salt	anti-oxidant effects: ↑vit. E, GSH, ↓MDA, and tendency to ↓GSSG and 4-HHE in plasma pro-oxidant effects: small ↓plasma GSH-Px and GSSG-R activity endocannabinoid effects: ↓AEA and LEA in plasma	[129]
CBD 10 mg/kg, *i.p.*, once daily, 14 days	SHR	anti-oxidant effects: ↓CO gr., tendency to ↑GSH, ↓GSSG, and 4-HNE in plasma pro-oxidant effects: small ↓plasma GSH-Px activity endocannabinoid effects: ↓SEA, HEA, DGLEA and tendency to ↓PEA, OEA, LEA in plasma other effects: ↓free AA in plasma	[129]
		KIDNEY	
AEA 3 mg/kg, *i.v.*, once daily, 14 days	Dahl salt-sensitive + high salt (8%) diet	pro-oxidant effects: ↓Nrf2 in renal cortex other effects: - ↑Smad3 in renal cortex and ↑interstitial fibrosis and glomeruli damage score - ↑Ca^{2+} excretion on day 7	[109]
PEA 30 mg/kg, *s.c.*, once daily, 5 weeks	SHR	vasodilatory effects: - ↑vasodilatory metabolites (HETEs and EETs) synthesis and/or ↓their degradation - ↓RAAS activity (↓AT_1R, ↑AT_2R signaling pathway) anti-oxidant and anti-nitrosative effects: - ↓ROS, MDA and ↑Cu-Zn-SOD and p47phox - ↓iNOS and protein nitrotyrosylation - small ↓urinary MDA and nitrite other effects: ↑urinary output - ↓severity of glomerulosclerosis and tubulointerstitial fibrosis	[107]

Table 4. *Cont.*

Compound, Dose, and Protocol	Model	Effects	References
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	DOCA-salt	anti-hypertrophic effects: ↓renal hypertrophy (only in younger rats) anti-oxidant effects: ↓ROS, XO, NADPH oxidase, Trp and ↑GSH-Px, GSSG-R activity, ↑GSH, vit. A, p-cJun, ↓Keap1 pro-oxidant effects: ↓Cu-Zn-SOD, CAT activity and ↑4-HNE, MDA, 8-OHdG and ↓p21 and HO-1 anti-inflammatory effects: ↓TNFα and ↓COX-1 and COX-2 activity endocannabinoid effects: ↓FAAH and MAGL activity - ↑AEA, 2-AG, and NADA; ↓CB_1R, ↑ CB_2R, and TRPV1 other effects: ↑free AA, DHA, and phospholipid AA - intensification of changes induced by hypertension	[119,142,143]
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	SHR	anti-oxidant effects: ↓ROS, XO, CO gr.; ↑Cu-Zn-SOD activity, GSH, vit. E, A, HO-1 pro-oxidant effects: ↓GSH-Px activity, ↑4-HNE, MDA, NPs, 8-OHdG, Keap1, Bach1, ↓p21 anti-inflammatory effects: ↓COX-1, COX-2 activity pro-inflammatory effects: ↑cPLA2 activity endocannabinoid effects: ↓FAAH and MAGL activity - ↑AEA, 2-AG, and NADA; ↑CB_2R and CB_1R other effects: ↑free AA and DHA - prevention of changes in electrical properties of the cell membrane, sialic acid, and protein content	[142,143]
rimonabant 10 mg/kg, oral, once daily, 4 weeks	(mRen2)27	other effects: ↑urine osmolality (at day 21)	[127]
		LIVER	
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	DOCA-salt	anti-oxidant effects: ↓XO, NADPH oxidase, ↑Cu-Zn-SOD, GSH-T activity, ↑GSH, GSSG, vit. A, ↓Trp, Keap1, Bach1, ↑p-cJun pro-oxidant effects: ↓GSSG-R activity, vit. E, p21, ERK1/2, HO-1, ↑4-HNE, MDA, 4-ONE, 8-OHdG, dityrosine anti-inflammatory effects: ↓NFκB, TNFα endocannabinoid effects: ↓FAAH and MAGL activity - ↓2-AG, ↑ CB_1R, and ↓ PPARα other effects: ↓phospholipid DHA and LA - ↓ apoptosis (↓caspase 3, 9 but ↑ caspase 8)	[113]
URB597 1 mg/kg, *i.p.*, twice daily, 14 days	SHR	anti-oxidant effects: ↓XO, NADPH oxidase, ↑CAT, GSH-Px activity, p21, p-ERK1/2, HO-1, ↓ CO gr. pro-oxidant effects: ↓GSSG-R activity, ↑MDA, 8-OHdG, Keap1, Bach1, ↓ p-cJun, Trx anti-inflammatory effects: ↓NFκB, TNFα, and ↑COX-2 endocannabinoid effects: ↓FAAH activity - ↑AEA, NADA, ↓ CB_2R, and ↑TRPV1 other effects: ↓phospholipid AA, free AA, and ↑ free DHA, LA	[144]

Table 4. *Cont.*

Compound, Dose, and Protocol	Model	Effects	References
Δ^8-THC, Δ^9-THC 3 mg/kg, *i.p.*, once daily, 14 days	ARH unilaterally adrenalectomized +1% NaCl [1]	hypertrophic effects: ↑liver hypertrophy/weight	[132]

The Table summarizes all significant effects described in particular publications. Non-significant results are not mentioned. [1] Female animals. ↑ increase; ↓ decrease; *i.c.v.*—intracerebroventricular; *i.p.*—intraperitoneal; *i.v.*—intravenous; *s.c.*—subcutaneous; 2-AG—arachidonoylglycerol; 4-HHE—4-hydroxyhexenal; 4-HNE—4-hydroxynonenal; 4-ONE—4-oxononenal; 8-OHdG—8-hydroxy-2′-deoxyguanosine; AA—arachidonic acid; AEA—anandamide; ARH—adrenal regeneration hypertension; AT_1R—angiotensin II type 1 receptor; AT_2R —angiotensin II type 2 receptor; Bach1—transcription regulator protein BACH1; CAT—catalase; CB_1R—cannabinoid receptor type 1; CB_2R—cannabinoid receptor type 2; CBD—cannabidiol; CO gr.—protein carbonyl groups; COX—cyclooxygenase; cPLA2—cytosolic phospholipase A2; Cu-Zn-SOD—cytosolic superoxide dismutase; DGLEA—dihomo-γ-linolenoyl ethanolamide; DHA—docosahexaenoic acid; DOCA—deoxycorticosterone acetate; EETs—epoxyeicosatrienoic acids; ERK—extracellular signal-regulated kinases; FAAH—fatty acid amide hydrolase; FGIR—fasting glucose/insulin ratio; GPR—G protein-coupled receptor; GSH—glutathione; GSH-Px—glutathione peroxidase; GSH-T—glutathione transferase; GSSG—glutathione disulfide; GSSG-R—glutathione reductase; HEA—homo-γ-linolenyl ethanolamide; HETEs—hydroxyeicosatetraenoic acids; HO-1—heme oxygenase 1; HOMA-IR—homeostasis model assessment of insulin resistance; Hsp70—70 kilodalton heat shock protein; IL—interleukin; iNOS—inducible nitric oxide synthase; Keap1—kelch-like ECH-associated protein 1; LA—linoleic acid; LEA—linolenoyl ethanolamide; MAGL—monoacylglycerol lipase; MDA—malondialdehyde; NADA—N-arachidonoyl dopamine; NADPH—nicotinamide adenine dinucleotide phosphate; nf-AEA—nanoformulated anandamde; NF-κB—nuclear factor kappa-light-chain-enhancer of activated B cells; NO—nitric oxide; NPs—neuroprostanes; Nrf2—nuclear factor erythroid 2-related factor 2; OEA—oleoyl ethanolamide; p-cJun—phosphorylated transcription factor Jun; p21—cyclin-dependent kinase inhibitor 1; p47phox—neutrophil cytosolic factor 1; PC—phosphatidylcholine; PE—phosphatidylethanolamine; PEA—palmitoyl ethanolamide; PI—phosphatidylinositol; PPAR—peroxisome proliferator-activated receptors; PS—phosphatidylserine; QUICKI—quantitative insulin sensitivity check index; RAAS—renin-angiotensin-aldosterone system; ROS—reactive oxygen species; RVLM—rostral ventrolateral medulla; SEA—stearoyl ethanolamide; SHR—spontaneously hypertensive rat; Smad3—mothers against decapentaplegic homolog 3; THC—tetrahydrocannabinol; TNFα—tumor necrosis factor α; Trp—tryptophan; TRPV1—transient receptor potential vanilloid 1; Trx—thioredoxin; TUNEL—terminal deoxynucleotidyl transferase dUTP nick end labeling; uCRP—ultrasensitive C-reactive protein; vit.—vitamin; WT-1—Wilms' tumor-1 transcription factor; XO—xanthine oxidase.

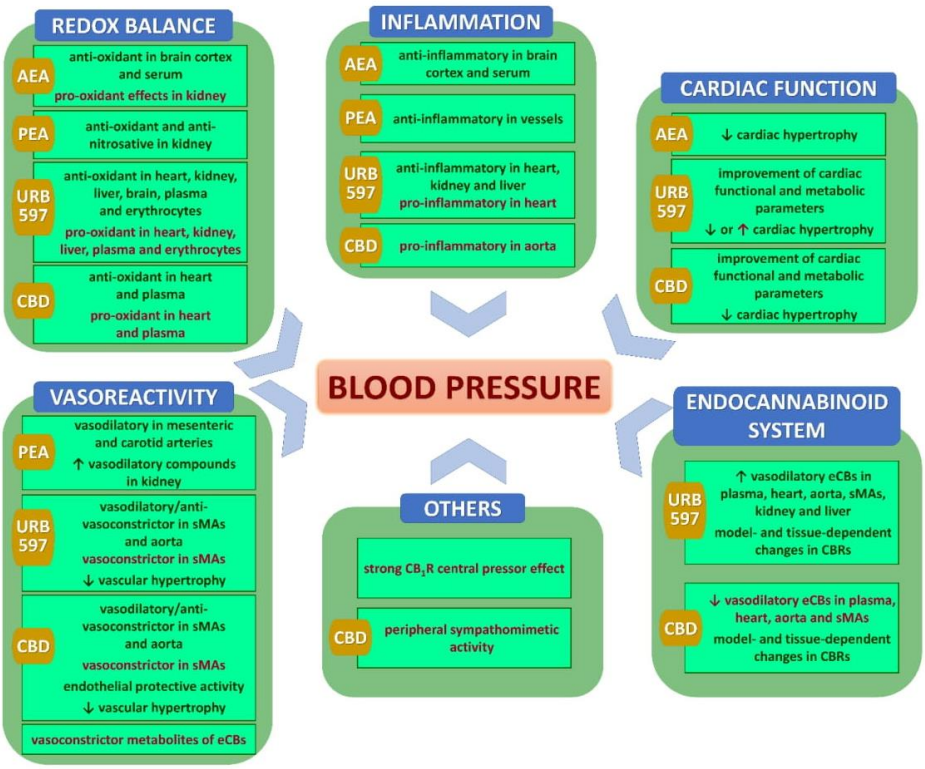

Figure 2. Summarized effects of multitarget (endo)cannabinoids on blood pressure. For clarity, the effects, listed in detail in Tables 3 and 4, are partly simplified and are based on results of all parameters connected with particular mechanisms, which sometimes were opposite. ↑—increase; ↓—decrease; AEA—anandamide; CBRs—cannabinoid receptors; CB_1R—cannabinoid type 1 receptor; CBD—cannabidiol; eCBs—endocannabinoids; PEA—palmitoyl ethanolamide; sMAs—small mesenteric arteries.

10.1. Vasodilatation

The strong vasodilating effects of (endo)cannabinoids in isolated vessels, depending on the hypertension model, have been described (Section 7 and Table 2). Notably, chronic administration of (endo)cannabinoids enhanced some vasorelaxant action (mostly in resistance arteries) via the following mechanisms: (1) improvement of the vasodilator effect elicited by Ach and/or MethAEA observed after chronic treatment with PEA [108] and URB597 [95] in SHR, and CBD in both DOCA-salt and SHR [94]; (2) reduction in vasoconstrictor response to phenylephrine in DOCA-salt [101] and SHR [95] under chronic FAAH inhibition; (3) enhancement of vasodilating compound synthesis (such as epoxyeicosatrienoic acids (EET), NO, and PGI_2) or decrease in RAAS activity in vessels [94,108]; and (4) decrease in aortic hypertrophy and/or sMAs in SHR and DOCA-salt hypertensive animals treated with URB597 or CBD [94,95,101].

On the contrary, in some cases, pro-constrictive effects were observed, such as increased vasoconstriction induced by thromboxane A_2 analog or decreased response of the vasorelaxant sodium nitroprusside (SNP) after chronic URB597 [95] and CBD [94] administration, respectively, observed in sMAs of SHR. These effects may at least partially counteract the compounds' beneficial effects on hypertension.

10.2. Cardiac Functional Antihypertensive Effects

Several beneficial changes in cardiac functional parameters were noted after chronic cannabinoid treatment: (1) decreased diastolic stiffness after URB597 in DOCA-salt [116]

and CBD in SHR [130], (2) improved cardiostimulatory isoprenaline influence (positive inotropic and lusitropic effects under chronic URB597 [116] and CBD [130] treatment, respectively), (3) normalized cardiac negative inotropic effect of CB_1R agonist CP55940 (only in DOCA-salt rats) after both URB597 and CBD, and (4) diminished carbachol-induced vasoconstriction of coronary arteries after chronic CBD administration in DOCA-salt and SHR [130]. In addition to the functional improvements, cannabinoids were potent in diminishing left ventricle (LV) overgrowth, the most prominent hypertrophic effect of systemic hypertension. The effectiveness was demonstrated by nf-AEA [110], URB597 [116,121], and CBD [130]. A similar anti-hypertrophic effect was observed in the kidneys of DOCA-salt animals treated with URB597 [119]. Since many place the kidney at the center of the pathobiology of systemic hypertension [8], this could be the reason for the better reaction to URB597 treatment in DOCA-salt.

10.3. Changes in Endocannabinoid System Components

The hypotensive effect or lack of an effect may also be induced by changes in eCBs released in different tissues. eCBs with proven vasodilating properties were characterized before (see Section 7).

As shown in Tables 3 and 4, changes in eCBs distribution have been studied after chronic treatment with URB597 and CBD only. The effectiveness of treatment was confirmed by decreased FAAH activity in various tissues, as well as for CBD, which inhibits this enzyme [49]. URB597 also diminished MAGL activity in the heart, mesenteric artery, kidney, and liver of DOCA-salt and/or SHR (Tables 3 and 4). Using these two hypertension models allowed us to demonstrate that changes in the levels of eCBs and their receptors are mainly tissue- and model-dependent. URB597 acted more uniformly than CBD. It mostly increased the levels of potentially vasorelaxant eCBs in plasma (AEA and NADA in DOCA-salt and SHR; 2-AG in SHR), heart (AEA in SHR; NADA and 2-AG in DOCA-salt and SHR), aorta (AEA and 2-AG in SHR), sMAs (AEA in SHR), kidneys (AEA, 2-AG, NADA in both models), or liver (AEA and NADA in SHR). In contrast, CBD mainly decreased eCB levels in the heart (2-AG, OEA) and plasma (AEA) in DOCA-salt and plasma (small PEA, OEA) in SHR. In the aorta, it also reduced NAGly levels in DOCA-salt and AEA in SHR, and tended to diminish levels of 2-AG, PEA, and NAGly in SHR. On the other hand, it increased concentrations of AEA, 2-AG, PEA, and DEA in the aortas of DOCA-salt animals. In the case of CBD, changes in the levels of other compounds with so far unknown vasodilatory potentials, such as DEA, DGLEA, LEA, EPEA, DHEA, HEA, and 2-LG, in various tissues of hypertensive animals have been determined.

Besides activating or blocking various receptors, cannabinoids may self-regulate their action by altering the expression of classical and non-classical CBRs in the tissues. As shown in Figure 1, activation of those receptors should result in beneficial effects, so an increase in expression is considered positive and a decrease negative. A different situation occurs where CB_1Rs are concerned because they may evoke both protective and damaging processes. After URB597 treatment of DOCA-salt rats, an increase in CB_2Rs and TRPV1 (heart, kidney), GPR55, and PPARα (heart) and a decrease in CB_1Rs (kidney, tendency in LV) were observed. On the other hand, an increase of CB_1Rs in the heart and liver and a decrease of PPARα in the liver and PPARγ receptors in the heart occurred [113,116,117,142]. Quite different changes happened in the SHR model. The expression of CB_2R (heart, kidney), GPR55 (heart, brain), TRPV1 (liver), and PPARγ (heart) receptors increased, and CB_1R decreased in the aorta but increased in the heart and kidney, whereas the expression of CB_2R (liver, brain), TRPV1 (heart), and PPARα (heart) receptors decreased [117,140,142,144]. Chronic administration of CBD also elicited model-dependent changes in receptor expression. CB_1R expression decreased in the heart and sMAs, but increased in the aorta; CB_2R expression decreased in the heart but increased in sMAs and aorta; and GPR18 decreased in the heart in DOCA-salt animals. In SHR, CB_1R expression decreased in the heart but increased in sMAs and aorta, CB_2R expression increased in sMAs and aorta, GPR18 decreased in the heart, and TRPV1 increased in the aorta [94,129]. To

summarize, as listed above, the effects of URB597 and CBD on the expression of various receptors are tissue- and model-dependent. However, it seems that, in general, beneficial effects dominate over negative ones.

10.4. Anti- and Pro-Oxidative Effects

Known anti- and pro-oxidative effects of activation/blockade of CBRs (see Figure 1), as well as direct inhibitory action of CBD affecting oxidative and nitrosative stress [145], implicate them as possible mechanisms involved in the regulation of BP [8,62].

Indeed, as shown in Tables 3 and 4, depending on the administration protocol and hypertension model, AEA caused anti-oxidant effects in CNS and serum (less frequent administration of the nanoformulated form in SHR) [111] and pro-oxidants in the kidney (frequent *i.v.* dosing in Dahl salt-sensitive animals) [109]. In these two cases, post-treatment oxidative status corresponded to changes in BP, i.e., decrease and increase, respectively. In contrast to AEA, a pronounced anti-oxidant effect of PEA in the kidney is postulated as one of the main mechanisms responsible for the pressure drop following chronic administration of this compound [107].

Chronic URB597 administration caused ambiguous oxidative effects in hypertension (Tables 3 and 4). In both DOCA-salt and SHR, it resulted in almost the same intense pro- and anti-oxidative impact on heart tissue [117,120], which was also confirmed in rat plasma [117], erythrocytes [141], kidney [142], and liver [113,144]. The only clear anti-oxidant effect was observed in the SHR brain [140], which did not lead to a fall in BP (small or no antihypertensive effect; Table 3).

CBD, well known for its anti-oxidant (mostly direct) properties [145], showed not unequivocal but rather positive modifications in the redox balance of hypertensive rats [129]. However, given the lack of an antihypertensive effect, the outcome was either too weak or counteracted by other opposing effects.

10.5. Anti-Inflammatory Effects

Inflammation is also inextricably linked to oxidative stress in hypertension [8]. As shown in Tables 3 and 4, chronic (endo)cannabinoid administration exerts mainly anti-inflammatory effects. Unfortunately, inflammatory parameters have been examined relatively rarely. Importantly, anti-inflammatory consequences in hypertension support previously described anti-oxidant effects of PEA (mesenteric bed) [108] and nf-AEA (CNS and serum) [111]. URB597 treatment mostly showed effects against inflammation in cardiac tissue [120], kidney [142], and liver [113,144]. The use of CB_1R antagonists [128] or CB_2R agonists [106] also resulted in decreased inflammation (in the aorta and CNS, respectively), which could explain the hypotensive effect of the above compounds. Importantly, it was demonstrated recently that marijuana smoking elevated plasma markers of inflammation associated with atherosclerosis and that THC-induced inflammation, oxidative stress, and endothelial dysfunction in mice were responsive to the CB_1R antagonist genistein [139].

10.6. Other Pro-Hypertensive Effects

The mechanisms described above do not always fully explain the presence or absence of the hypotensive effect of (endo)cannabinoids. The question arises as to what other factors, sometimes only literature-based, could reduce the potential hypotensive effects of chronically administered compounds.

One factor could be central CB_1Rs, activation of which is responsible for the pressor effect. As mentioned in Section 8, *i.v.* injection of (endo)cannabinoids decreased BP in anesthetized animals but increased it in conscious animals. Microinjection of (endo)cannabinoids into the PVN enhanced BP in anesthetized and conscious rats, and chronic administration of the CB_1R antagonist rimonabant decreased BP (Table 2). These three effects suggest that the central mechanisms responsible for the increased BP induced by cannabinoids may be superior to those involved in hypotension (at least in some models of hypertension).

Another aspect that should be noted is that acute *i.v.* injection of CBD strongly increased SBP and HR but decreased DBP in pithed rats (a model that allows examination of peripheral effects only since the animals' CNS is destroyed). Enhancement of both of these cardiovascular parameters was evoked by the peripheral sympathomimetic activity of CBD; the lower DBP was probably related to the direct vasodilatory properties of CBD. Two opposite effects are probably responsible for CBD at 10 mg/kg not affecting cardiovascular parameters within 1 h after *i.p.* administration in conscious rats [146].

It should also be kept in mind that the well-known vasodilatory action of eCBs may sometimes be diminished by their vasoconstrictor metabolites, e.g., OEA [58] and AEA, which is even suggested as a PH enhancer (for details, see Section 12) [102]. Similarly, 2-AG can act differently on the vessels (through vasodilation or vasoconstriction) [48,58] and can also have opposite effects on the heart (protective or damaging) [147,148].

11. Why Multitarget Vasodilatory (Endo)cannabinoids Are Not Effective as Antihypertensive Compounds

To summarize, Tables 3 and 4 show the effects of chronic administration of monotarget (rimonabant, LH-21, JWH133, and O-1602) and multitarget (PEA, AEA, URB597, JZL195, CBD, and THC) (endo)cannabinoids on systemic hypertension. We included O-1602 in the monotarget group since it has a higher affinity for GPR55 than GPR18 receptors [149], and other multitarget compounds act by at least three different targets (e.g., CBD, 65 targets) [150]. Except for CBR antagonists and inhibitors of enzymes responsible for eCB degradation, all compounds possess proven vasodilatory properties, in many cases also in hypertension (Table 2 and Section 7), and were shown to decrease BP more strongly in anesthetized hypertensive rats than normotensive rats after acute *i.v.* administration (Section 8). It should be emphasized that all monotarget (endo)cannabinoids are synthetic ones. Among multitarget compounds, synthetic, phyto-, and endocannabinoids can be found. (Endo)cannabinoid origin (synthetic, plant-derived, or endogenous) is not, therefore, an indicator of its potential beneficial action in hypertension.

Chronic administration of all monotarget substances caused a significant fall in BP. However, experiments were conducted on only one model of hypertension in each study. What is more, very specific routes of administration (*i.a.* for O-1602, *i.c.v.* for JWH133), rather impossible to translate into human therapy, were used. In addition, a clinical trial of rimonabant in obese patients was conducted, in which an extracted group of individuals with hypertension showed decreased BP with the compound. Still, it is not certain whether the effect was due to weight loss. Besides, rimonabant was withdrawn from the market due to serious side effects [39].

The results considering chronic administration of multitarget (endo)cannabinoids are more complicated. AEA increased or decreased BP, URB597 caused a small, model-dependent drop in BP or had no hypotensive effect, and CBD failed to modify BP regardless of the model used. Only PEA clearly decreased BP in SHR. However, this effect was noticed only in the fifth week of administration. Interestingly, similar to PEA, a delayed hypotensive response was observed with the other compounds (for details, see Table 3), which rather excludes the direct influence of vasodilatation as the main reason for their influence on BP.

Figure 2, which outlines various influences of multitarget compounds on BP in hypertension, is an attempt to answer the main question of why multitarget vasodilatory (endo)cannabinoids are not effective as antihypertensive compounds. They can lead to a fall in BP as a result of not only direct vascular relaxation but also the release of various vasorelaxant compounds, the enhancement of such action elicited by other endogenous substances (e.g., Ach), the release of vasodilatory eCBs or decreased vasoconstrictor activity (e.g., phenylephrine), and reduced cardiac and vessel hypertrophy and anti-oxidant and anti-inflammatory capacity in various tissues.

However, chronic AEA, URB597, or CBD administration can also stimulate effects leading to increased BP. First of all, it should be kept in mind that (endo)cannabinoids produce complex cardiovascular effects and that central CB_1Rs are also responsible for

stimulating the distinct pressor response (for details, see Section 6). AEA is a potent CBR agonist. CBD, well known as a negative allosteric modulator of CB_1Rs, can also stimulate this receptor. Recently, central CB_1Rs have been demonstrated as a target in CBD action in anxiety, in a manner sensitive to rimonabant and absent in $CB_1^{-/-}$ mice [150]. Moreover, eCBs can also cause vasoconstriction via their metabolites. Additionally, the model- and tissue-dependent influence on sensitivity to cannabinoid receptors might also determine the direction of changes in BP since stimulation of CB_1Rs enhances oxidative and inflammatory states (see Figure 1). Thus, after chronic URB597 and CBD treatment, some pro-vasoconstriction changes were observed. Importantly, the anti-oxidant activity of these two compounds was accompanied by an almost equally intense pro-oxidative effect. URB597 also showed a slight pro-inflammatory effect, partly interfering with its overall anti-inflammatory properties. The same is true for CBD, a known anti-inflammatory compound, which showed minor inflammatory activity. In the case of CBD, two additional observations should be taken into consideration: (1) it reduced the level of vasodilatory eCBs; (2) it possesses peripheral sympathomimetic activity (for details, see Section 10.6). Finally, the model- and tissue-dependent influence on sensitivity to cannabinoid receptors might also determine the direction of changes in BP since stimulation of CB_1Rs enhances oxidative and inflammatory states (see Figure 1).

In summary, monotarget compounds seem more beneficial as potential antihypertensive drugs than multitarget compounds. In this context, synthetic monotarget cannabinoids should have an advantage over endocannabinoids, which do not have such precise sites of action. However, monotarget compounds were examined in one hypertension model only, specific routes of administration (*i.a.* or *i.c.v.*) were used, and the CB_1R antagonist rimonabant, which had been examined in long-term clinical studies, was withdrawn from the market because of its undesirable side effects. Thus, further experiments with monotarget cannabinoids are needed to determine the best compounds. The first single experiments with agonists of CB_2 and GPR55 receptors and with a peripheral CB_1R antagonist are encouraging. The bad experience with rimonabant excludes the recommendation of other first-generation CB_1R antagonists (that cross the blood–brain barrier), although central CB_1Rs responsible for the pressor effect seem to strongly counteract the peripheral vasodilatory effect anyway. In light of this, the third generation of CB_1R antagonists, i.e., peripherally restricted dual-target CB_1R antagonists (e.g., hybrid CB_1R antagonist and inducible NOS inhibitor) [39], remains to be examined.

12. In Vivo Effects of Chronic (Endo)cannabinoids in PH

As shown in Table 2 of the review by Krzyżewska et al. [48], all main components of the endocannabinoid system (AEA, 2-AG, CB_1Rs, CB_2Rs, TRPV1, GPR18, GPR55 receptor, and FAAH) are present in the pulmonary circulation or lung tissue. Importantly, eCBs AEA, 2-AG, virodhamine, the endogenous agonists of GPR55 (l-alpha-lysophosphatidylinositol (LPI)) and GPR18 (NAGly) receptors caused full or almost full relaxation of pre-constricted human pulmonary arteries [48].

However, in contrast to its potent vasodilatory activity, AEA is postulated to mediate hypoxia-induced pulmonary vasoconstriction [102] based on the following facts: (1) hypoxia stimulated AEA synthesis in pulmonary arterial smooth muscle cells in vitro; (2) AEA (but not 2-AG) increased pulmonary arterial tone in isolated perfused mouse lungs via its vasoconstrictor metabolites (Table 2); (3) genetic FAAH deletion or chronic administration of FAAH inhibitor URB597 prevented the onset of PH (Table 5). The beneficial influence of FAAH inhibition could result from the inhibition of vasoconstrictor metabolite synthesis or the enhancement of AEA and its protective action, neither of which was determined under in vivo conditions. Notably, the vasoconstriction effect of AEA on isolated perfused mouse lungs was more pronounced in female animals (Table 2), which is in line with the statistic that PH is more common in women.

As shown in Table 5 and Figure 3, except for the paper by Wenzel et al. mentioned above, the chronic effects of (endo)cannabinoids on PH have only been examined in the last

two years. Importantly, all those studies revealed the positive effects of the administered drugs. First of all, there was a significant decrease in right ventricular systolic pressure (RVSP), the main parameter determining the severity of the disease. This is very interesting since the authors used different, sometimes contrary, targets. As mentioned above, FAAH inhibition prevented PH development [102]. On the other hand, the peripheral CB_1R antagonist JD5037 alone tended to lower RVSP only in the MCT-induced model of rat PH. Still, it potentiated the effect of metformin in a combined therapy protocol [151]. Thus, the roles of AEA and CB_1Rs remain to be examined in detail.

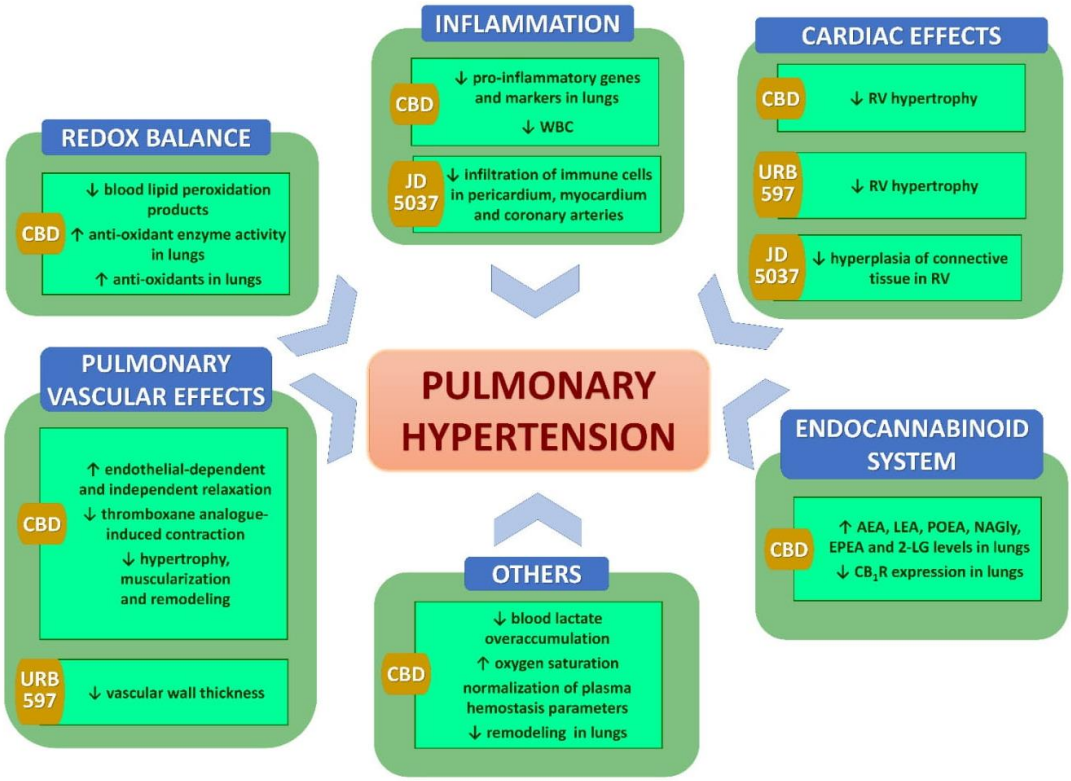

Figure 3. Summarized effects of (endo)cannabinoids on pulmonary hypertension. For clarity, the effects, listed in detail in Table 5, are partly simplified. ↑—increase; ↓—decrease; 2-LG—2-linoleoylglycerol; AEA—anandamide; CB_1R—cannabinoid type 1 receptor; CBD—cannabidiol; EPEA—eicosapentaenoyl ethanolamide; LEA—linolenoyl ethanolamide; NAGly—N-arachidonoyl glycine; PA—pulmonary artery; POEA—palmitoleoyl ethanolamide; RV—right ventricle; WBC—white blood cells.

The richest data available are for phytocannabinoid CBD. It has been used in two models of PH, the Sugen/hypoxia mouse model [152] and the rat MCT model [152,153]. Two protocols were applied: 14-day treatment or 21-day preventive in the former, and 21-day preventive in the latter. In both, CBD caused a strong drop in RVSP. Comparable effects of CBD in CB_2R knockout mice and their wild-type littermates confirmed the lack of involvement of those receptors in its protective action [152]. In addition, CB_1Rs were found to not participate in the anti-PH activity of CBD [48].

All experiments investigating chronic cannabinoids in PH showed anti-hypertrophic effects of the compounds (Table 5). The most common were decreased Fulton's index, which indicates hypertrophy of RV induced by increased afterload and reduced vascular hypertrophy. CBD also altered PA reactivity (intensified response to relaxants and

diminished response to constrictors) [153]. The mechanism of action was examined in more detail for CBD only. Protection against changes induced by PH might be based on anti-inflammatory or anti-oxidant action in blood and lungs [152,153]. Additionally, CBD increased pulmonary levels of some eCBs with vasodilatory effects on PA [153]. Furthermore, studies on PH reported an influence on systemic BP in both normotensive and PH groups.

As with systemic hypertension, studies mostly used routes of administration that are convenient (*i.p.*, *i.g.*), but this would not fully meet the expectations of possible future clinical practice. An interesting solution in the case of PH would be administration by inhalation [137,138]. This could produce not only a systemic response but also (or maybe only) a local effect in the lung tissue, which is known to be the center of the disease. Importantly, treatment delivered by inhalation is already being used in therapy for PAH (treprostinil), with a good isolated effect on pulmonary vasculature [2]. On the other hand, results obtained in a randomized controlled trial demonstrated that single-dose inhalation of vaporized cannabis did not modify the airway function in patients with advanced chronic obstructive pulmonary disease (COPD) [154].

In summary, CBD appears more effective against pulmonary than systemic hypertension (see Section 9). The question is how to explain it. In both types of hypertension, the authors used the same dose (10 mg/kg; a higher dose was not better in PH studies) and a similar route of administration (*i.p.*; intragastric (*i.g.*) only in experiments on mice). The potential beneficial effect of CBD on systemic hypertension was examined only with the use of a therapeutic (14-day) protocol, while for PH, both therapeutic (14-day) and preventive (21-day) protocols were used. The therapeutic scheme used might be the reason for the lack of the compound's effectiveness in systemic hypertension since it is more difficult to reverse disease progression than to prevent its development. Interestingly, the effects of CBD in systemic hypertension were model-dependent, while a comparable influence of CBD in two PH models was observed. It should be kept in mind that the pulmonary and systemic vasculature have uniquely distinct roles and features; the pulmonary circulation is a low-resistance, high-capacity circuit with the advantage of local regulatory mechanisms, whereas systemic blood vessels are high-resistance, low-capacity conduits. In addition, the peripheral sympathomimetic effect of CBD determined in systemic hypertension (see Section 10.6) may not play an important role in PH since it was mainly observed as a cardiac component (increased HR) which was not observed in PH models.

Table 5. Effects of chronic administration of cannabinoids on various tissues of male animals (unless noted otherwise) in different models of pulmonary hypertension.

Compound, Dose, and Protocol	Model	Effects			Ref.
CARDIOVASCULAR					
			Influence on Changes Induced by Hypertension		
		BP and HR Effects	**Cardiac Effects/Expression in Heart (If Not Stated Otherwise)**	**Vascular Effects**	
$FAAH^{-/-}$ in comparison to WT	hypoxia (mice) [1]	- no ↑RVSP	hypertrophic effects: no ↑Fulton index	hypertrophic effects: no ↑vascular wall thickness	[102]
URB597 5 mg/kg, *i.p.*, once daily, 3 days or 3 weeks		- ↓RVSP (in longer procedure) (by ~5 mmHg)	anti-hypertrophic effects: ↓Fulton index (in longer procedure)	anti-hypertrophic effects: ↓vascular wall thickness (in longer procedure)	
JD5037 3 mg/kg, oral, once daily, 3 weeks	MCT (rat)	- intensification of the metformin-induced ↓RVSP - ↔BP; ↔HR	anti-hypertrophic effects: ↓hyperplasia of connective tissue in myocardium anti-inflammatory effects: - ↓infiltration of immune cells in pericardium, myocardium, and coronary arteries other effects: - ↓vacuolization of tunica media of coronary arteries	-	[151]
CBD 10 mg/kg, 20 mg/kg, *i.g.*, once daily, 14 days (treatment) or 3 weeks (preventive)	SuHx/ SuHx *Cnr2*$^{-/-}$ (mice)	- ↓RVSP (by ~10 mmHg)	anti-hypertrophic effects: ↓Fulton index	anti-hypertrophic effects: - ↓PA hypertrophy - ↓PA muscularization - ↓remodeling ($PCNA^+$/nuclei)	[152]
CBD 10 mg/kg, *i.p.*, once daily, 3 weeks (preventive)	MCT (rat)	- ↓RVSP (by ~15 mmHg) - ↔BP; ↔HR	anti-hypertrophic effects: small ↓Fulton index	vasodilatory effects in PA: - ↑endothelial-dependent (Ach) and endothelial-independent (SNP) relaxation - ↓thromboxane analog-induced contraction anti-hypertrophic effects in PA: - ↓hypertrophy - ↓muscularization - ↓remodeling ($PCNA^+$/nuclei)	[152,153]

Table 5. *Cont.*

Compound, Dose, and Protocol	Model	Effects			Ref.
CARDIOVASCULAR					
			Influence on Changes Induced by Hypertension		
		BP and HR Effects	**Cardiac Effects/Expression in Heart (If Not Stated Otherwise)**	**Vascular Effects**	
BLOOD					
CBD 10 mg/kg, *i.g.*, once daily, 3 weeks (preventive)	SuHx (mice)		anti-oxidant effects: ↓blood MDA other effects: ↓blood lactate overaccumulation		[152]
CBD 10 mg/kg, *i.p.*, once daily, 3 weeks (preventive)	MCT (rat)		anti-inflammatory effects: ↓WBC other effects: - ↑oxygen saturation - normalization of plasma hemostasis parameters (↓PAI-1 and t-PA levels)		[153]
LUNGS					
CBD 10 mg/kg, *i.g.*, once daily, 3 weeks (preventive)	SuHx (mice)		anti-oxidant effects: ↑GSSG-R and GSH-Px activity anti-inflammatory effects: ↓*Il6* and *Tnfα* other effects: ↓lactate accumulation (↓*Pfkfb3*)		[152]
CBD 10 mg/kg, *i.p.*, once daily, 3 weeks (preventive)	MCT (rat)		anti-oxidant effects: ↑TAC, GSH, GSSG-R activity anti-inflammatory effects: ↓NFκB, TNFα, MCP-1, IL-1β, CD68 endocannabinoid effects: - ↑AEA, LEA, POEA, NAGLy, EPEA, and 2-LG; ↓CB_1R other effects: ↓Gal-3		[153,155,156]

The Table summarizes all significant effects described in particular publications. Non-significant results are not mentioned. [1] Female animals. ↑ increase; ↓ decrease; ↔ no effect; *i.g.*—intragastrical; *i.p.*—intraperitoneal; 2-LG—2-linoleoylglycerol; Ach—acetylcholine; AEA—anandamide; BP—blood pressure; CB_1 R—cannabinoid receptor type 1; CBD—cannabidiol; CD68—cluster of differentiation 68; *Cnr2*$^{-/-}$—knockout of gene encoding CB_2 R protein; EPEA—eicosapentaenoyl ethanolamide; FAAH—fatty acid amide hydrolase; Gal-3—galectin 3; GSH—glutathione; GSH-Px—glutathione peroxidase; GSSG-R—glutathione reductase; HR—heart rate; IL—interleukin; *Il6*—gene encoding IL-6 protein; LEA—linolenoyl ethanolamide; MCP-1—monocyte chemoattractant protein-1; MCT—monocrotaline; MDA—malondialdehyde; NAGLy—N-arachidonoyl glycine; NF-κB—nuclear factor kappa-light-chain-enhancer of activated B cells; PA—pulmonary artery; PAI-1—plasminogen activator inhibitor 1; PCNA—proliferating cell nuclear antigen; *Pfkfb3*—gene encoding 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 enzyme; POEA—palmitoleoyl ethanolamide; RVSP—right ventricular systolic pressure; SNP—sodium nitroprusside; SuHx—sugen/hypoxia model; t-PA—tissue plasminogen activator; TAC—total antioxidant capacity; TNFα—tumor necrosis factor α; *Tnfα*—gene encoding TNFα protein; WBC—white blood cells; WT—wild type.

13. Conclusions

Our review summarizing publications regarding chronic administration of (endo) cannabinoids in experimental models of hypertension demonstrates that the best outcomes in systemic hypertension were obtained using a few monotarget compounds. In contrast, chronic administration of multitarget (endo)cannabinoids failed to modify higher BP, and they are not recommended for the treatment of systemic hypertension since they induce responses leading to both decreased and increased BP (for details, see Figure 2).

The best results in PH were obtained with chronic administration of CBD (the only compound examined in detail), which was effective in two PH models and two treatment protocols (preventive and therapeutic). Since significant differences exist between the systemic and pulmonary vasculature and the pathophysiology of systemic and pulmonary hypertension, it seems reasonable to examine other (endo)cannabinoids (including multitarget) against PH.

Importantly, in chronic preclinical experiments on normo- and hypertension, (endo) cannabinoids were found to be rather safe compounds, with no serious adverse effects (except in the aggressive AEA *i.v.* administration protocol), so they can be used for other indications.

To summarize, other preclinical and clinical studies are still needed to determine the beneficial role of vasodilator (endo)cannabinoids in systemic (only monotarget) or pulmonary (both mono- and multitarget) hypertension.

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Chapter 10.

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Article

Chronic Cannabidiol Administration Fails to Diminish Blood Pressure in Rats with Primary and Secondary Hypertension Despite Its Effects on Cardiac and Plasma Endocannabinoid System, Oxidative Stress and Lipid Metabolism

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Abstract: We investigated the influence of cannabidiol (CBD) on blood pressure (BP) and heart rate (HR) in spontaneously (SHR) and deoxycorticosterone (DOCA-salt) hypertensive rats. Hypertension was connected with increases in cardiac and plasma markers of lipid peroxidation in both models, whereas cardiac endocannabinoid levels decreased in SHR and increased in DOCA-salt. CBD (10 mg/kg once a day for 2 weeks) did not modify BP and HR in hypertension but counteracted pro-oxidant effects. Moreover, it decreased cardiac or plasma levels of anandamide, 2-arachidonoylglycerol and oleoyl ethanolamide in DOCA-salt and inhibited the activity of fatty acid amide hydrolase (FAAH) in both models. In the respective normotensive control rats, CBD increased lipid peroxidation, free fatty acid levels and FAAH activity. In conclusion, chronic CBD administration does not possess antihypertensive activity in a model of primary and secondary (DOCA-salt) hypertension, despite its antioxidant effect. The latter may be direct rather than based on the endocannabinoid system. The unexpected CBD-related increase in lipid peroxidation in normotensive controls may lead to untoward effects; thus, caution should be kept if CBD is used therapeutically.

Keywords: 2-arachidonoylglycerol; anandamide; cannabidiol; cannabinoid receptor; SHR; DOCA-salt; endocannabinoids; oxidative stress

1. Introduction

Cannabidiol (CBD) is one of the most abundant cannabinoids derived from the *Cannabis sativa* plant and devoid of a psychoactive effect [1,2]. CBD binds to cannabinoid CB_1 and CB_2 receptors with much lower affinity than Δ^9-tetrahydrocannabinol (THC) [3] and interacts with GPR18, GPR55 and TRPV1 receptors [4]; it possesses a very marked antioxidant effect [5–7]. CBD is licensed for the

treatment of some types of childhood epilepsy (Dravet and Lennox-Gastaut syndrome) in the United States [4,8] and, in combination with THC, for the treatment of multiple sclerosis-associated spasticity in Canada and in the European Union [4]. In addition, a potential therapeutic action of CBD is being considered in anxiety disorders, schizophrenia, depression, Alzheimer's disease, Parkinson's disease, pain, cancer, inflammatory and autoimmune diseases and diabetic complications [2,4,9].

CBD may become a strategy also for the treatment of cardiovascular diseases, including hypertension [3,9]. To date, blood pressure-lowering effects of CBD were observed under stress conditions in humans [10–12] and in stressed animals [13,14]. However, the effect of CBD on the blood pressure of hypertensive individuals has been studied in one study only; in a paper on conscious spontaneously hypertensive rats [15], a single intraperitoneal dose of CBD (10 mg/kg) failed to affect blood pressure.

Hypertension is a disease with a complex pathomechanism, which includes, among others, changes in the endothelium and redox balance, both within the heart and blood vessels [16,17]. CBD is suggested to be a potential positive modulator of hypertension thanks to its vasodilatory action [3,9,11,18]. Another property that may be of key importance in a potential antihypertensive activity of CBD is its impact on oxidative stress. Attenuation of oxidation and/or nitration parameters by CBD was observed in acute experiments on human endothelium cells treated with high glucose [19], on the liver of mice subjected to ischemia/reperfusion [20] and on mouse hippocampal cells subjected to oxygen plus glucose deprivation/reperfusion [21]. Similar beneficial effects were also obtained in chronic experiments on the heart [22] and retina [23] from diabetic mice, on mouse hepatic cells with ethanol-induced liver injury [24,25], on the heart from doxorubicin-treated mice [26] and rats [27] and on the heart and other tissues of rats with sepsis [28].

The mechanism of CBD in the latter studies is complex and probably results from direct antioxidant properties [3,29] but may also be related to an effect on the endocannabinoid system, which is important for the modulation of oxidative stress [30,31]. CB_1 receptors are mainly associated with its promotion [32–34], whereas CB_2 [35–39] and GPR18 [40,41] receptors reduce oxidation parameters in cardiovascular system including heart. There are contradictory reports regarding modulation of oxidative stress by TRPV1 and GPR55 receptors [30,31].

Although CBD probably does not work via endocannabinoid receptors directly, it may act through augmentation of endocannabinoid tone [42]. CBD inhibits fatty acid amide hydrolase (FAAH) [43] and can interact with the anandamide membrane transporter [44,45] both of which may increase levels of endocannabinoids and related lipids. They may have positive effects and be used as a target in pharmacotherapy [46] but in some cases, can also exert untoward actions [47]. In this context, one should keep in mind that the FAAH inhibitor URB597 and hypertension may affect cardiac and plasma oxidative stress, endocannabinoid levels and lipid metabolism in a model-dependent manner [48,49].

The first aim of this study was to investigate whether chronic, unlike acute [15], administration of CBD reduces blood pressure (BP) and heart rate (HR) in rats with primary and secondary hypertension. Moreover, we studied whether CBD has an impact (ii) on the redox system, (iii) the endocannabinoid system in heart and plasma and (iv) free polyunsaturated fatty acids (PUFAs) and phospholipid PUFAs.

2. Results

2.1. General

As shown in Table 1 and Figure 1 (in which cardiovascular parameters were measured by the non-invasive method and telemetrically, respectively) SBP and DBP, registered before the first administration of CBD or its vehicle, were higher in SHR and DOCA rats than in the respective control animals (WKY and SHAM). HR tended to be lower in DOCA compared to SHAM rats and higher in SHR than in WKY when non-invasive registration was used (Table 1); in animals with telemetrical registration, HR was higher in WKY than in SHR (Figure 1). Two-week administration of CBD 10 mg/kg did not affect SBP, DBP and HR in normo- or hypertensive rats. The vehicle for CBD decreased (or tended to decrease) HR by about 7%–8% in WKY and SHAM. Similarly CBD

reduced HR by about 10% in WKY (Table 1). We did not observe any cardiovascular effect of CBD during 24-h observation on the first and the last day of its administration (Figure 1).

Table 1. Influence of cannabidiol (CBD) or its vehicle on systolic blood pressure, heart rate and body weight in conscious spontaneously (SHR) and deoxycorticosterone (DOCA) hypertensive rats and their respective normotensive controls (Wistar-Kyoto (WKY) and sham-operated (SHAM) rats).

Group	Treatment	*n*	Systolic Blood Pressure (mmHg)		Heart Rate (Beats/min)		Body Weight (g)	
			Before the First Dose of CBD or Its Solvent	24 h After the Final Dose of CBD or Its Solvent	Before the First Dose of CBD or Its Solvent	24 h After The Final Dose of CBD or Its Solvent	Before the First Dose of CBD or Its Solvent	24 h After the Final Dose of CBD or Its Solvent
WKY	vehicle	7	111 [95;122]	98 [91;105] !	341 [321;366]	314 [304;326]	342 [329;361]	380 [367;400] !!!
WKY	CBD	7	101 [87;124]	92 [87;107]	334 [313;357]	300 [298;321] !	325 [325;340]	360 [353;390] !
SHR	vehicle	7	178 [161;199] ***	174 [164;187] ***	359 [351;369]	367 [365;387] **	290 [282;292] ***	300 [296;326] ***!
SHR	CBD	7	184 [172;192] $$$	172 [170;176] $$$	355 [352;397]	358 [344;380] $$	315 [300;325]	328 [313;340] $!!!
SHAM	vehicle	7	122 [111;132]	120 [118;131]	363 [351;371]	336 [331;340]!	294 [270;304]	320 [302;345] !!!
SHAM	CBD	7	111 [100;129]	121 [109;134]	354 [333;374]	349 [328;371]	278 [256;294]	322 [286;340] !
DOCA	vehicle	7	163 [111;175]	175 [160;190] ***	314 [303;360]	324 [312;352]	275 [233;276]	280 [252;321]
DOCA	CBD	6	150 [143;174] $	173 [150;189] $$$	327 [315;354]	314 [301;348]	280 [271;298]	303 [297;312] !

Cardiovascular parameters were measured by the non-invasive tail-cuff method. CBD (10 mg/kg) or its vehicle were injected intraperitoneally once daily for 14 days. Data are expressed as the medians with interquartile range; $/! $p < 0.05$; **/$$ $p < 0.01$; ***/$$$/!!! $p < 0.001$ significantly different from the respective values obtained in normotensive groups receiving vehicle for CBD (*) or CBD ($) or recorded before the first dose of the compound (!).

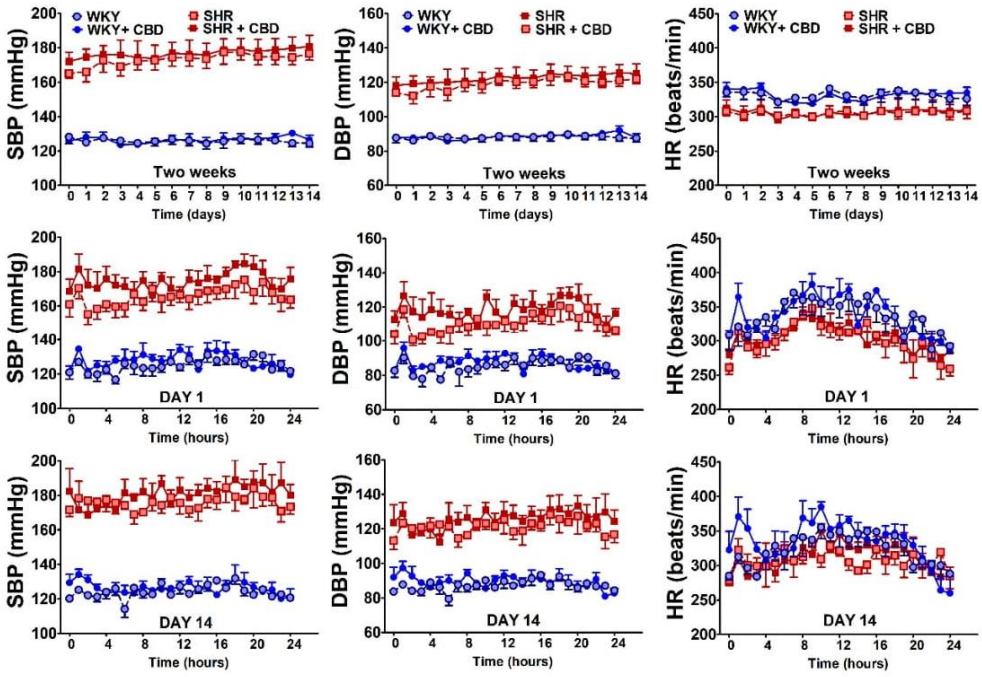

Figure 1. Influence of cannabidiol (CBD) or its vehicle on systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) in spontaneously hypertensive rats (SHR) and their normotensive controls (WKY). Parameters were measured telemetrically every hour (shown for DAY 1 and 14 in the intermediate and bottom panels, respectively) and means for each day were determined (shown in the top panels). CBD (10 mg/kg) was injected i.p. once daily for 14 days (control animals received the vehicle for CBD instead); first and final injection on DAY 1 and 14, respectively. Data are expressed as the median with interquartile range; $n = 4$. All values of SBP and DBP in SHR (in the presence of CBD or its vehicle) were higher than their respective values in WKY ($p < 0.001$), and values of HR in SHR were lower than in WKY ($p < 0.001$ with 2 exceptions at the 14th day: WKY vs. SHR $p < 0.05$ and WKY + CBD vs. SHR + CBD: $p < 0.01$).

The body weight of SHR and DOCA rats was lower than that of WKY and SHAM rats, respectively, both before the first and after the final administration of the vehicle of CBD (Table 1). When animals were considered which had received CBD instead, differences were smaller or did not occur at all (Table 1).

2.2. Influence of Hypertension and CBD on the Endocannabinoid System

As shown in Figure 2, 2-AG showed the highest level among the endocannabinoids; values in the heart of normotensive WKY and SHAM rats (expressed as median (interquartile range)) amounted to 5.0 (4.7;7.5) ($n = 7$) and 1.3 (1.1;1.7) nmol/g tissue ($n = 7$), respectively. The levels of the best-known endocannabinoid AEA were relatively low, i.e., about 220 and 75 times lower than the respective values of 2-AG in WKY and SHAM rats. Although levels of PEA, LEA and OEA were higher than those of AEA, they were still lower than 2-AG levels by factors of about 15, 40 and 60 (WKY) and 5, 20 and 25 (SHAM), respectively. HEA, DEA and DGLEA showed the lowest levels, i.e., about 1–3 pmol/g tissue in all cases.

Hypertension modified cardiac endocannabinoid levels in a model-dependent manner (Figure 2). Thus, they mostly decreased (2-AG by 24% and LEA by 51%) or tended to decrease (AEA, DHEA POEA, HEA, DEA and DGLEA) in SHR in comparison to WKY. In contrast, they increased (2-AG by 158%, LEA by 97%, OEA by 180%, DHEA by 56%, DEA by 40% and DGLEA by 54%) in DOCA hearts in comparison to SHAM. Chronic CBD administration did not modify the cardiac endocannabinoid

levels in SHR and WKY. However, it decreased levels of 2-AG (−57%), OEA (−28%), DEA (−15%) and DGLEA (−20%) in hearts of DOCA but increased PEA (+68%) and HEA (+80%) levels in SHAM rats.

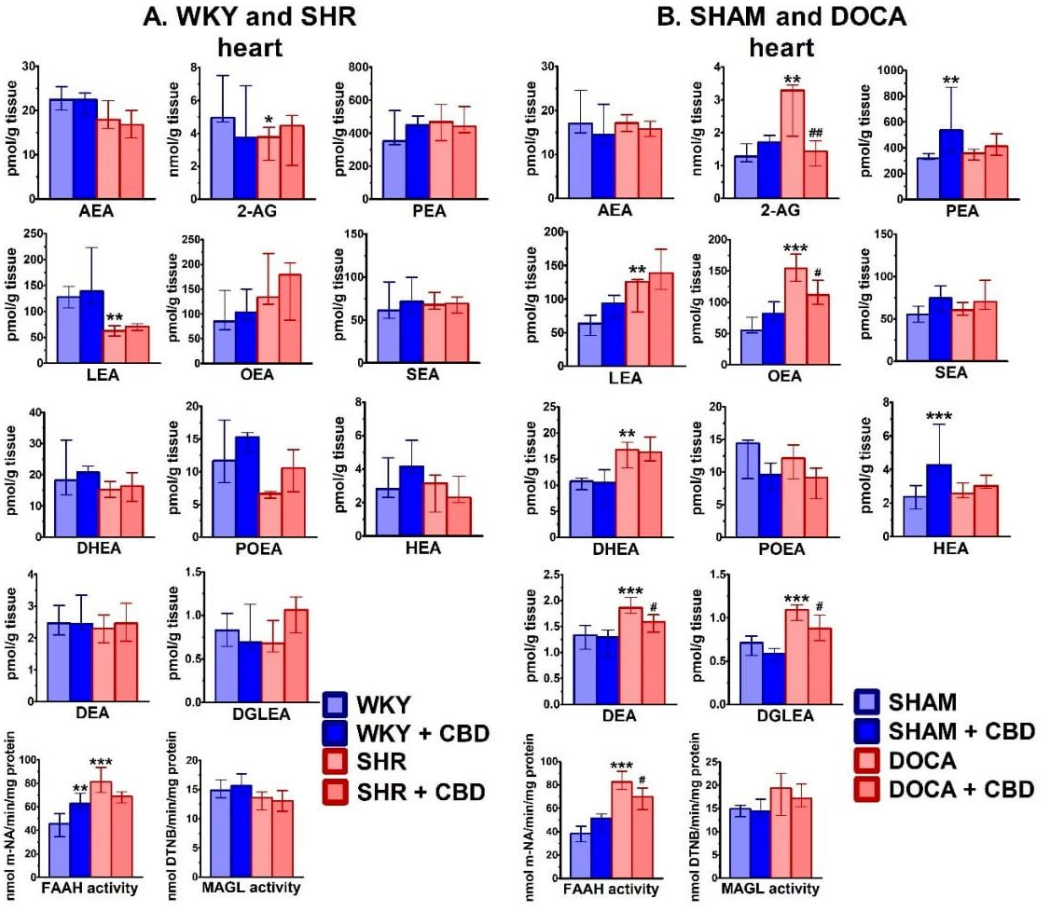

Figure 2. Influence of hypertension and cannabidiol (CBD) or its vehicle on endocannabinoid levels in heart isolated from spontaneously (SHR; **A**) and deoxycorticosterone-salt (DOCA; **B**) hypertensive rats and their normotensive controls (WKY (**A**) and SHAM (**B**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead; hearts were prepared 24 h after the final injection. Data are expressed as the median with interquartile range; n = 5-7; */# $p < 0.05$; **/## $p < 0.01$; *** $p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#). AEA—anandamide; 2-AG—2-arachidonoylglycerol; PEA—palmitoyl ethanolamide; LEA - linolenoyl ethanolamide; OEA—oleoyl ethanolamide; SEA—stearoyl ethanolamide; DHEA—docosahexaenoyl ethanolamide; POEA—palmitoleoyl ethanolamide; HEA—homo-γ-linolenyl ethanolamide; DEA—docosatetraenoyl ethanolamide; DGLEA—dihomo-γ-linolenoyl ethanolamide; FAAH—fatty acid amide hydrolase; MAGL—monoacylglycerol lipase; m-NA—m-nitroaniline; DTNB—5,5′-dithiobis-2-dinitrobenzoic acid.

In both models of hypertension, we observed increases in FAAH activities in comparison to the respective normotensive control (+77% in SHR and +115% in DOCA), but no changes in MAGL activities (Figure 2). CBD did not modify MAGL activity but decreased FAAH activity in DOCA and tended to do so in SHR. Moreover, it increased FAAH activity in WKY.

Plasma AEA levels were about 1 nM both in WKY and SHAM rats (Figure 3). For both control groups, levels were higher (PEA and OEA by about 10 and 5 times, respectively) and similar (SEA) when compared to the values of AEA. In contrast to SHR hearts, the plasma endocannabinoid levels increased (SEA +38%, DGLEA +63%) or tended to increase (OEA, POEA DHEA and DEA) in

comparison to WKY. In DOCA, plasma levels increased (AEA +38% and LEA +113%), decreased (OEA −29% and DHEA −41%) or tended to decrease (POEA and DEA) in comparison to SHAM rats.

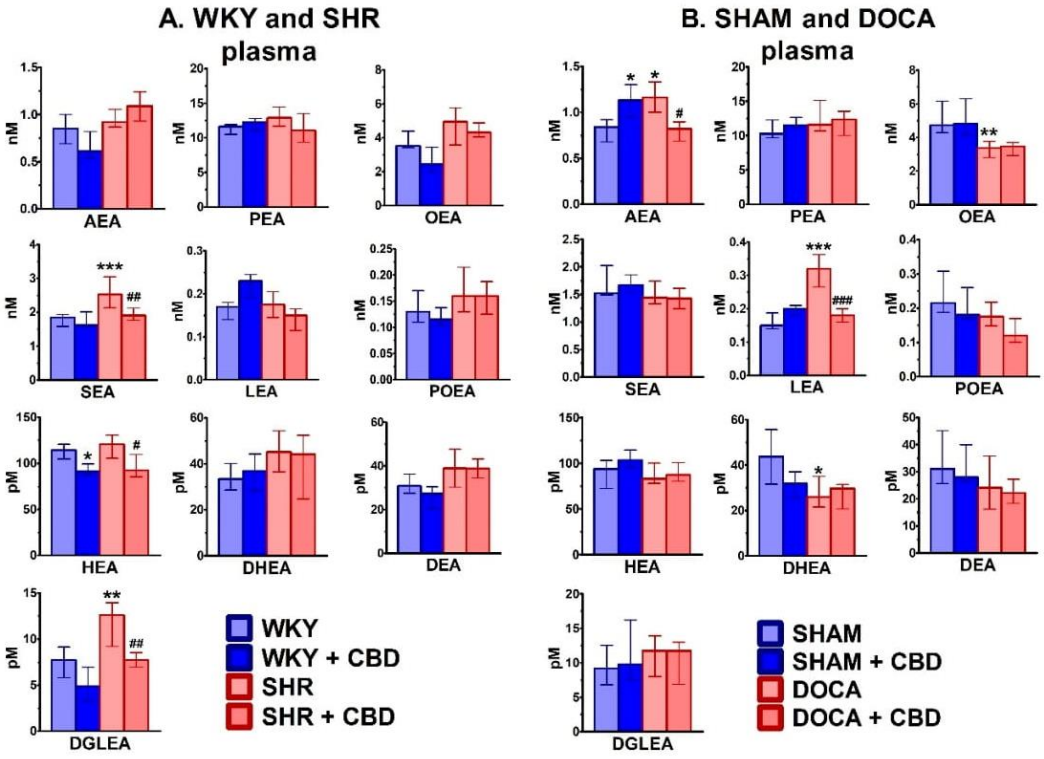

Figure 3. Influence of hypertension and cannabidiol (CBD) or its vehicle on endocannabinoid levels in plasma isolated from spontaneously (SHR; **A**) and deoxycorticosterone-salt (DOCA; **B**) hypertensive rats and their normotensive controls (WKY (**A**) and SHAM (**B**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead. Plasma was obtained 24 h after the final injection. Data are expressed as the median with interquartile range; n = 5–7; */# $p < 0.05$; **/## $p < 0.01$; ***/### $p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#). AEA—anandamide; PEA—palmitoyl ethanolamide; OEA—oleoyl ethanolamide; SEA—stearoyl ethanolamide; LEA—linolenoyl ethanolamide; POEA—palmitoleoyl ethanolamide; HEA—homo-γ-linolenyl ethanolamide; DHEA—docosahexaenoyl ethanolamide; DEA—docosatetraenoyl ethanolamide; DGLEA—dihomo-γ-linolenoyl ethanolamide.

Chronic CBD administration decreased plasma levels of SEA (−25%), HEA (−23%) and DGLEA (−39%) in SHR and AEA (−29%) and LEA (−44%) in DOCA or tended to do so in the case of PEA, OEA and LEA in SHR (Figure 3). Moreover, CBD decreased plasma levels of HEA (−20%) in WKY and increased the plasma level of AEA (+33%) in SHAM or tended to decrease levels of AEA, OEA, POEA, DEA and DGLEA in WKY and levels of POEA and DHEA in SHAM.

In the cardiac left ventricle, cannabinoid CB_1 and CB_2 receptor density decreased but GPR18 and GPR55 density increased in SHR compared to WKY. In DOCA, compared to SHAM, only a decrease in CB_1 density and an increase in GPR18 density occurred. The increase in GPR18 receptor density was particularly marked in both hypertension models (by about 60%) (Figure 4). Chronic CBD administration decreased GPR55 density in SHR and tended to decrease CB_1 and GPR18 receptor density in SHR and CB_1, CB_2 and GPR18 receptor density in DOCA. Moreover, CBD decreased CB_1 and tended to reduce GPR18 receptor density in WKY. TRPV1 receptor density was not modified by hypertension or CBD treatment (Figure 4).

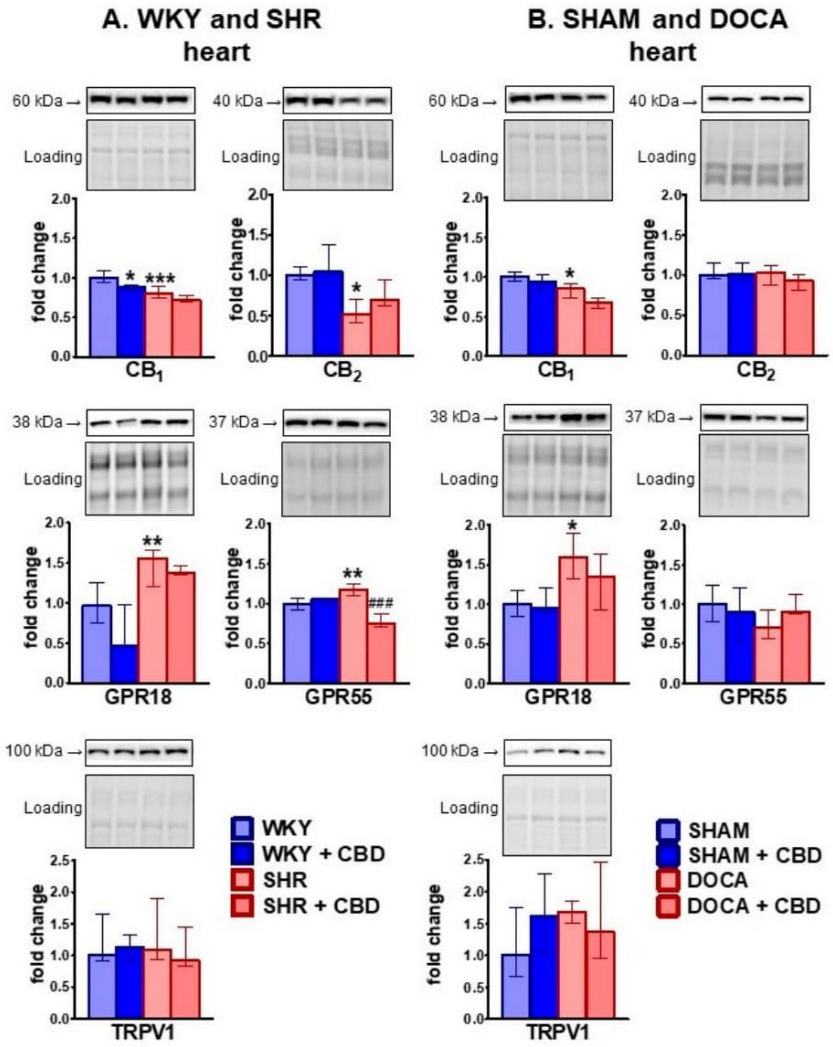

Figure 4. Influence of hypertension and cannabidiol (CBD) or its vehicle on expression of receptors in left ventricle isolated from spontaneously (SHR; **A**) and deoxycorticosterone-salt (DOCA; **B**) hypertensive rats and their normotensive controls (WKY (**A**) and SHAM (**B**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead. Hearts were prepared 24 h after the final injection. Receptor protein was determined by Western blots and given as fraction of the value in the normotensive control (first of the four columns). Images obtained using stain-free gel technology that allows for total protein visualization and quantification are shown as a loading control (Loading). Data are expressed as the median with interquartile range; $n = 5–6$; * $p < 0.05$; ** $p < 0.01$; ***/### $p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#).

2.3. Influence of Hypertension and CBD on Oxidative Stress

As shown in Figure 5, activities of cardiac antioxidant principles underwent slight changes in hypertension. Thus, in comparison to the respective normotensive tissues, GPx activity increased or tended to increase in DOCA (+48%) and SHR, respectively, whereas GSR activity decreased (−12%) in DOCA heart. However, SOD and CAT were not altered in SHR and DOCA. The cardiac vitamin A (but not E) level increased in SHR (+198%), whereas the levels of both antioxidant vitamins did not change in the heart of DOCA rats. Levels of glutathione and glutathione disulfide were altered by

both models of hypertension in the same way: GSH decreased in the heart of SHR (−30%) and DOCA (−42%) and GSSG increased (+82% and +149%, respectively). Products of lipid peroxidation increased in the heart of SHR (4-HNE +45% and 4-HHE +86%) and DOCA rats (MDA +82%). Changes in cardiac CO groups were neither observed in SHR nor in DOCA.

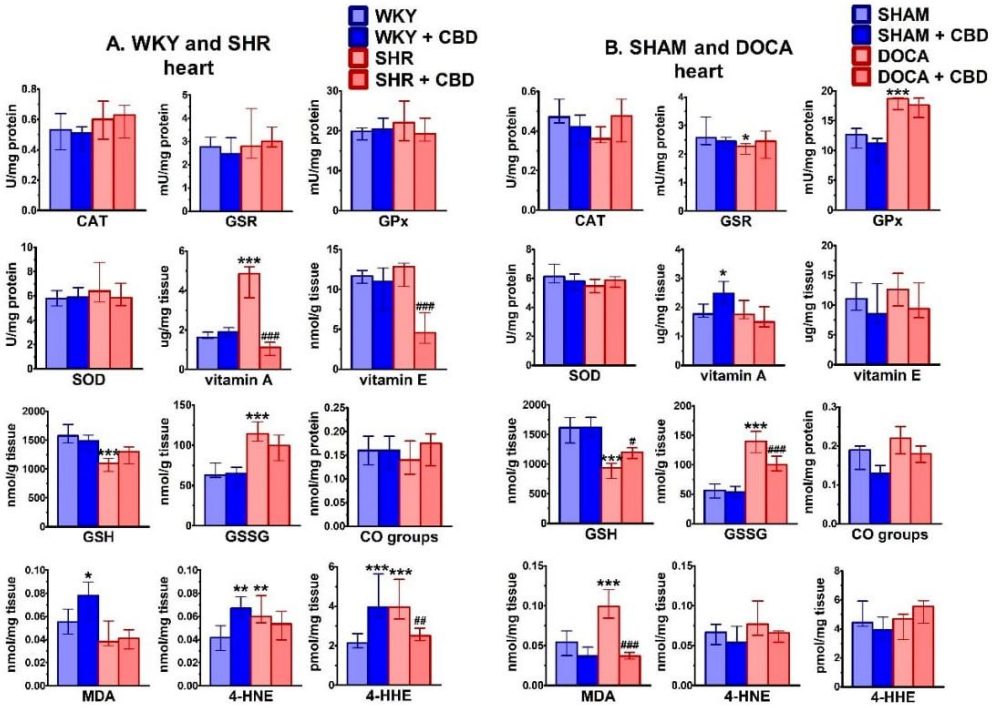

Figure 5. Influence of hypertension and cannabidiol (CBD) or its vehicle on activity/level of oxidative stress parameters in heart isolated from spontaneously (SHR; **A**) and deoxycorticosterone-salt (DOCA; **B**) hypertensive rats and their normotensive controls (WKY (**A**) and SHAM (**B**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead. Hearts were prepared 24 h after the final injection. Data are expressed as the median with interquartile range; n = 5–7; */$^{\#}$ $p < 0.05$; **/$^{\#\#}$ $p < 0.01$; ***/$^{\#\#\#}$ $p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#). CAT—catalase; GSR—glutathione-disulfide reductase; GPx—glutathione peroxidase; SOD—superoxide dismutase; GSH—glutathione; GSSG—glutathione disulfide; CO groups—protein carbonyl groups; MDA—malondialdehyde; 4-HNE—4-hydroxynonenal; 4-HHE—4-hydroxyhexenal.

Chronic CBD administration was associated mainly with a decrease in cardiac levels of the products of lipid peroxidation (Figure 5). The effect was significant for 4-HHE (−37%) in SHR and for MDA (−63%) in DOCA and a tendency was observed in the case of 4-HNE in SHR and DOCA. Moreover, cardiac levels of vitamin A (−77%) and E (−64%) were reduced in SHR or tended to be so in DOCA. In addition, the level of GSH was increased (+29%) and that of GSSG was decreased (−29%) in DOCA; in SHR, both parameters tended to be altered Other cardiac parameters of oxidative stress were not modified by CBD in hypertensive rats (Figure 5). In normotensive rats, CBD increased levels of vitamin A in SHAM (+41%) and unexpectedly levels of products of lipid peroxidation (MDA (+42%), 4-HNE (+61%) and 4-HHE (+86%)) in WKY but not in SHAM (Figure 5).

Similarly to the heart, plasma activities of antioxidant principles underwent only small changes in hypertension in comparison to normotension (Figure 6). Thus, only in SHR SOD (+3%) and GPx activities increased or tended to increase, respectively. The level of vitamin E decreased in SHR (−65%), but was not modified in DOCA. Levels of vitamin A did not undergo changes in either hypertension model. Levels of GSH and GSSG were modified in a comparable way in SHR and DOCA, i.e., they decreased and increased by about 45% and 175%, respectively. Levels of MDA (but

not of other products of lipid peroxidation) increased by about 65% both in SHR and DOCA. The CO group level increased in SHR (+46%) and tended to do so in DOCA.

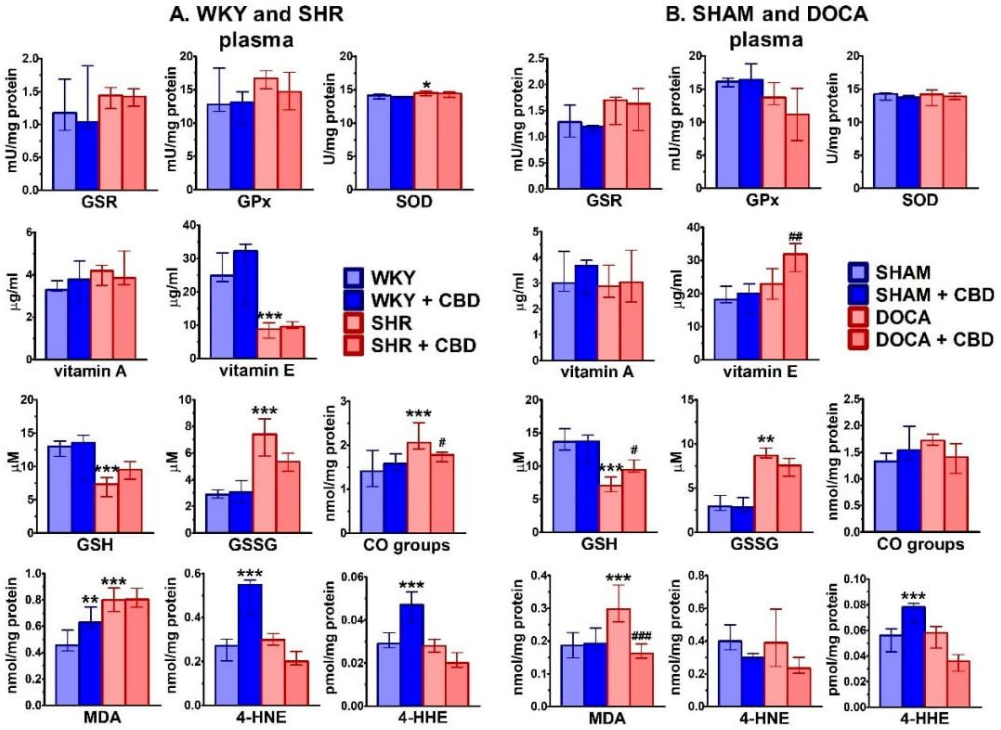

Figure 6. Influence of hypertension and cannabidiol (CBD) or its vehicle on activity/level of oxidative stress parameters in plasma isolated from spontaneously (SHR; **A**) and deoxycorticosterone-salt (DOCA; **B**) hypertensive rats and their normotensive controls (WKY (**A**) and SHAM (**B**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead. Plasma was obtained 24 h after the final injection. Data are expressed as the median with interquartile range; n = 5–7; $^{*/\#}p < 0.05$; $^{**/\#\#}p < 0.01$; $^{***/\#\#\#}p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#). GSR—glutathione-disulfide reductase; GPx—glutathione peroxidase; SOD—superoxide dismutase; GSH—glutathione; GSSG—glutathione disulfide; CO groups—protein carbonyl groups; MDA—malondialdehyde; 4-HNE—4-hydroxynonenal; 4-HHE—4-hydroxyhexenal.

Chronic CBD administration increased plasma levels of vitamin E (+39%) in DOCA. GSH was increased (+34%) in DOCA and tended to be increased in SHR whereas GSSG tended to decrease in either hypertensive rat model. For antioxidant enzyme activities only tendencies towards a decrease of GPx (SHR and DOCA) and GSR (DOCA) were observed. However, CBD reduced plasma levels of CO groups (-14%) in SHR and of MDA (-45%) in DOCA. Moreover, it tended to decrease plasma levels of 4-HNE (-32%) in SHR and 4-HHE (-37%) in DOCA. Unexpectedly it also increased plasma levels of MDA (+38%), 4-HNE (+101%) and 4-HHE (+62%) in WKY and of 4-HHE (+39%) in SHAM (Figure 6).

2.4. Influence of Hypertension and CBD on Lipids

The effect of hypertension on free fatty acids and phospholipids was studied in cardiac tissue (Figure 7A,B) and plasma (Figure 7C,D). Among FFA, hypertension increased and decreased levels of AA (+50%) and of LA (−51%) in the heart of DOCA, respectively, but did not modify cardiac levels of FFA in SHR and plasma levels both in SHR and DOCA. In the fraction of phospholipids, hypertension increased plasma levels of AA (+25%) and DHA (+48%) and tended to increase plasma

levels of LA in DOCA. On the other hand, it decreased cardiac levels of DHA (−25%) in SHR and LA (−28%) in DOCA and plasma levels of LA (−16%), AA (−42%) and DHA (−51%) in SHR.

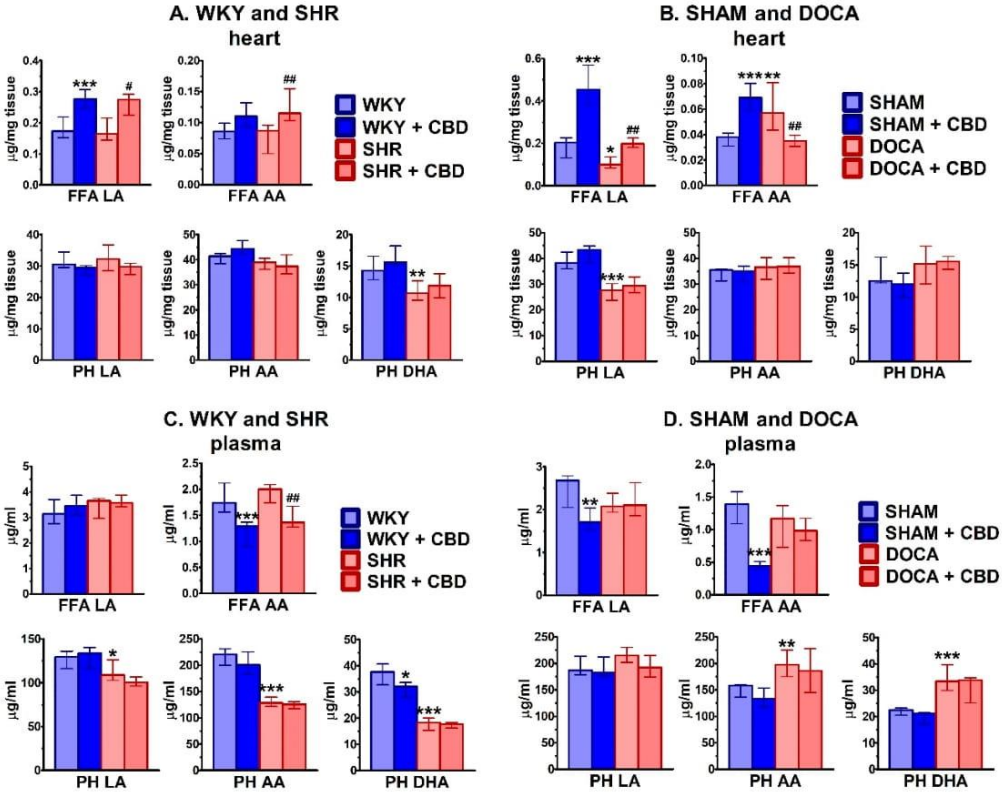

Figure 7. Influence of hypertension and cannabidiol (CBD) or its vehicle on levels of phospholipids (PH) and free fatty acids (FFA) in heart (**A**,**B**) and plasma (**C**,**D**) isolated from spontaneously (SHR; **A**,**C**) and deoxycorticosterone-salt (DOCA; **B**,**D**) hypertensive rats and their normotensive controls (WKY (**A**,**C**) and SHAM (**B**,**D**), respectively). CBD (10 mg/kg) was injected i.p. once daily for 14 days; the respective controls received the vehicle of CBD instead. Hearts were prepared and plasma was obtained 24 h after the final injection. Data are expressed as the median with interquartile range; n = 5–7; * $p < 0.05$; **/## $p < 0.01$; *** $p < 0.001$ significantly different from WKY or SHAM (*) and from SHR or DOCA (#). LA—linoleic acid; AA—arachidonic acid; DHA—docosahexaenoic acid.

Chronic CBD administration increased cardiac levels of FFA LA (+67%) and FFA AA (+32%) in SHR and FFA LA (+100%) in DOCA but decreased FFA AA in the heart of DOCA (−39%) and in the plasma of SHR (−32%). In normotensive controls, CBD increased or tended to increase cardiac levels of FFA LA (+60%) and FFA AA (+28%) in WKY and cardiac levels of FFA LA (+123%) and FFA AA (+82%) in SHAM. On the other hand, CBD decreased plasma levels of FFA AA (−26%) and PH DHA (−15%) in WKY and FFA LA (−36%) and FFA AA (-68%) in SHAM.

3. Discussion

3.1. General

This study shows that chronic CBD administration did not modify BP and HR in rats with primary (SHR) and secondary (DOCA-salt) hypertension in spite of the reduction of cardiac and plasma oxidative stress. It inhibited the FAAH activity in both hypertension models, but had opposite effects on cardiac levels of various endocannabinoids and endocannabinoid-related lipids (decrease in SHR vs. increase in DOCA). Unexpectedly, CBD increased lipid peroxidation in normotensive controls and this alteration may lead to untoward effects.

Our study is based on rats with primary (SHR; the most frequently studied genetic hypertensive model [17] and secondary hypertension (DOCA-salt [17]; because a salt-rich diet is one of the main lifestyle factors leading to hypertension). WKY and SHAM served as the respective normotensive controls and allowed us to detect potential undesirable effects of CBD. We used CBD at 10 mg/kg for 14 days since its chronic i.p administration had a beneficial effect on cardiovascular tissues (including decreases in oxidative stress) from diabetic (7 days; [50]) and septic rats (9 days; [28]) and from mice with diabetic (11 weeks; [22]) and doxorubicin-induced cardiomyopathy (5 days; [26]) and experimental autoimmune myocarditis (46 days; [51]). Note that CBD was used at ~ 10 mg/kg also in several acute experiments [10,13,15].

To examine the mechanism(s) of CBD action in hypertension, we considered parameters of oxidative stress (e.g., GSH and GSSG), of the endocannabinoid system (ECS) and of other components of lipid metabolism. With respect to the ECS, the two endocannabinoids AEA and 2-AG and the endocannabinoid-related lipids POEA, HEA, DEA, DGLEA, PEA, LEA, OEA, SEA and DHEA were determined [52]. The latter five and AEA are degraded by FAAH [53,54]. FAAH and MAGL activities and CB_1, CB_2, GPR18, GPR55 and TRPV1 receptor expression have been quantified as well. With respect to lipid metabolism free polyunsaturated fatty acids (PUFAs) including AA, LA and DHA and phospholipid PUFAs were determined.

The present results confirm and extend our previous observations [49] that in the heart, 2-AG has the highest concentration among the 11 endocannabinoids or endocannabinoid-related lipids. Its levels in WKY and SHAM were 220 and 75 times higher than the concentration of the best-known endocannabinoid AEA, respectively. This is an important observation in the light of recent publications that 2-AG in vivo worsens heart function after acute myocardial infarction [47], increases the severity of the cerebral blood flow deficit [55] or promotes atherogenesis [56] on the one hand but ameliorates inflammatory stress-induced insulin resistance in cardiomyocytes on the other [57]. Unfortunately, there are only limited data regarding the cardiovascular effects of endocannabinoids other than 2-AG and AEA. Thus, chronic administration of PEA (30 mg/kg for 5 weeks) or OEA (5 mg/kg for 7 days) improved rat myocardial function in doxorubicin-induced heart failure [58] and decreased BP and protected against kidney injury in SHR via inhibition of oxidative stress [59].

3.2. Effect of Hypertension

SBP was elevated in primary and secondary hypertension of animals in which BP was determined by the non-invasive (both models) and/or telemetric (SHR) method. With respect to SHR, both methods revealed an identical SBP (of about 170 mmHg) whereas HR differed. Compared to WKY, HR in SHR was higher or lower when the tail-cuff method or telemetry was used, respectively. A significant influence of restraint on HR determination by the non-invasive method (higher HR in SHR than in WKY) was described earlier [60]. In our previous study [49], DOCA-salt treatment over a period of 6 weeks resulted in a much higher SBP in DOCA than in SHR (about 220 vs. 185 mmHg, respectively). To avoid such a difference in the current study we reduced DOCA-salt treatment to 4 weeks (prior to the onset of the CBD treatment) which resulted in comparable SBP values on the final day of experiments in SHR and DOCA treated with vehicle (about 170 mmHg).

The elevated BP was connected with a redox imbalance, similarly to our previous papers [48,49]. Thus, GSSG increased and GSH decreased in heart and plasma of both hypertensive models. Other markers, including increased MDA, 4-HNE, 4-HHE and oxidative protein modifications (CO) occurred only in one hypertensive model and/or only in heart or plasma. In SHR (but not in DOCA) a decrease in plasma vitamin E and, contrarily to expectation, an increase in cardiac vitamin A was observed.

Cardiac FAAH activities increased in both models of hypertension like in our previous study [49]. Moreover, the present results show that in addition to AEA and 2-AG [49] also LEA, DHEA, POEA, HEA, DEA and DGLEA decreased (or tended to decrease) in the heart of SHR whereas in the heart of DOCA not only 2-AG but also LEA, OEA, DHEA, DEA and DGLEA increased. On the rule, changes in plasma endocannabinoid levels were similar (but less pronounced) with the exception that in DOCA OEA and DHEA levels decreased. Since numerous endocannabinoid-like compounds

were changed in hypertensive animals, future studies should clarify whether these compounds affect the cardiovascular system. As suggested previously [49], the enhanced levels of AEA, LEA, OEA and SEA (all of which are degraded by FAAH) in spite of higher FAAH activity might indicate that in the heart of the DOCA group endocannabinoid synthesis is favoured [61] and/or AEA transporter activity is decreased [62].

Cardiac CB_1 density decreased and GPR18 receptor density increased in both hypertension models whereas CB_2 receptor density decreased in SHR only. The results obtained with cannabinoid and cannabinoid-like receptors only partially conform to those in our previous study [49]; differences may result from the fact that (i) receptor densities were determined in left ventricle vs. whole heart, respectively, and (ii) the time period for induction of DOCA-salt hypertension lasted for 4 vs. 6 weeks, respectively. As mentioned in the Introduction, stimulation of CB_1 receptors enhances oxidative stress, whereas CB_2 and GPR18 receptors have an opposite effect. Accordingly, the hypertension-induced enhancement in oxidative stress does not fit well to the changes in receptor densities since in both hypertension models, a decreased pro-oxidative receptor (CB_1) was connected with an increased anti-oxidative receptor (GPR18).

The hypertension-related changes in lipids other than the endocannabinoids were dependent on the hypertension model. Similarly to endocannabinoids, mainly decreases in phospholipids (cardiac PH DHA and plasma PH LA, AA and DHA) were obtained in SHR. By contrast, in DOCA plasma phospholipid AA and DHA and cardiac FFA AA increased whereas cardiac FFA LA and PH LA decreased. Differences in particular cardiac and plasma PUFAs between SHR and DOCA were also observed in our previous study [49].

3.3. Effect of Chronic CBD in Hypertensive Animals

Chronic administration of CBD failed to modify BP and HR in both models of hypertension; this is in harmony with our previous study [15], in which a single CBD administration had no effect. One may argue that the dose of CBD was too low or the duration of its application too short; however, both parameters conformed to the conditions chosen in previous studies in which CBD had a beneficial effect (see 3.1). One may also argue that the effectiveness of CBD was lost during the study; however, FAAH (which is inhibited by CBD; [43]) was still reduced (DOCA) or tended to be reduced (SHR) 24 h after the final dose of CBD.

How can we explain the lack of an antihypertensive effect of CBD in hypertension? CBD does not only possess vasodilatory activity mainly shown on isolated vessels (for review, see [9]) but, according to our recent study in pithed SHR and WKY [15], at an intravenous dose of 10 mg/kg, it also exhibits sympathomimetic effects. Opposite cardiovascular effects might be at least partially responsible for the lack of a hypotensive effect of CBD in our study.

Hypertension is associated with an enhancement of oxidative stress ([16]; current study) and CBD is known for its antioxidant properties (for literature, see Introduction). Indeed, chronic CBD administration led to increases in GSH and decreases in GSSG both in heart and plasma in both hypertension models. Moreover, CBD counteracted parameters of lipid peroxidation including the enhanced cardiac and/or plasma 4-HNE and 4-HHE in SHR and the enhanced cardiac and plasma MDA and 4-HNE in DOCA. Moreover, CBD reduced the enhanced concentration of carbonyl groups in plasma. The increase in plasma vitamin E in DOCA was another beneficial effect of CBD although, contrarily to expectation, cardiac levels of vitamin A and E in SHR decreased.

The endocannabinoid system is overactivated in hypertension [63]. Chronic CBD administration failed to modify the densities of cannabinoid and cannabinoid-like receptors (the only exception was the decrease in cardiac GPR55 levels in SHR) but decreased cardiac levels of 2-AG, OEA, DEA and DGLEA in DOCA and plasma levels of AEA and LEA in DOCA and of SEA, HEA and DGLEA in SHR. On the rule, CBD reduced the levels of those ECBs the concentrations of which were enhanced in hypertension. Accordingly, CBD failed to modify cardiac endocannabinoid levels in SHR since only decreases were obtained. Surprisingly, the decreases in ECBs were observed in spite of the reduced FAAH activity. Due to the vasodilatory and/or hypotensive effects of AEA and OEA [64,65], the CBD-induced decrease in AEA and OEA levels appears to be unfavourable in hypertension

whereas the decrease in cardiac 2-AG concentration might be beneficial because worsening of cardiac function by 2-AG has been described recently [47].

The effect of CBD on PUFAs was again dependent on the hypertension model and on the level of PUFAs in hypertension. Hypertension-induced increases in PUFAs were observed in DOCA only and CBD decreased the cardiac FFA AA and tended to decrease the plasma PH AA and DHA.

3.4. Effect of Chronic CBD in Normotensive Animals

CBD is generally recognized as a safe drug [66]. Surprisingly, we have obtained some unexpected effects of chronic CBD administration in normotensive rats. The most untoward influence was the enhancement of lipid peroxidation documented by increases of heart and plasma MDA, 4-HNE and 4-HHE in SHAM and, in addition, of plasma 4-HHE in WKY. These alterations were connected with a decrease in cardiac (antioxidant) GPR18 receptor density in WKY and an increase (or the tendency of an increase) in cardiac FFA LA and FFA AA in WKY and SHAM. Our findings, although not unequivocal (since in plasma decreases in FFA AA (WKY and SHAM), FFA LA (SHAM) and HEA (WKY) occurred), are reminiscent of a previous study [28], in which CBD (10 mg/kg) administered to rats for 9 days led to an increase in carbonyl groups in lung and liver.

3.5. Limitations of the Study

The present investigation, in which a blood pressure-lowering effect of CBD could not be shown despite its antioxidant effect, was restricted to two models of hypertension studied in male rats. The possibility has to be considered that an antihypertensive effect of CBD was missing for the following reasons. (1) The duration of CBD administration might have been too short or its dose too low and an increase in the duration of its application or the use of a higher dose might have led to more evident changes, especially of the markers of oxidative stress and probably also of the level of blood pressure. (2) In our previous paper, the chronic administration of the FAAH inhibitor URB597 decreased BP in DOCA but not in SHR, in which BP before the first dose of URB597 was about 220 vs. 185 mmHg, respectively [67]. As mentioned above, in the present study, the period for the induction of DOCA hypertension was reduced from 6 to 4 weeks, which resulted in lower SBP (about 170 mmHg). We cannot exclude that the lack of effect of CBD is related to the shorter induction time and the lower blood pressure level in DOCA. (3) We used DOCA as a secondary model of hypertension. The possibility has to be considered that a model of hypertension connected with changes in the renin-angiotensin-aldosterone system (RAAS) (the activity of which is modified by the endocannabinoid system) might have revealed a blood pressure-lowering effect of CBD. Interestingly, opposite effects of a CB_1 receptor antagonist have been found in the cardiovascular system of SHR and of rats with a RAAS-dependent hypertension (for review, see [63]). (4) There are gender-specific differences in hypertension [17,68] and it would be interesting to extend our experiments to female rats. (5) There is no ideal animal model of human hypertension. However, the advantage of rat genetic models of hypertension is their similarity to the BP/hypertension phenotypes observed in patients and that SHR responds to the antihypertensive effects of almost all classes of drugs approved for treatment of hypertension. The DOCA-salt model connected with unilateral nephrectomy provides a reliable animal model that can develop severe hypertension with some features of human low-renin hypertension [17].

The mechanism of action for the antioxidant effect of CBD was not determined. This is not a trivial task since the compound has many different molecular effects [1,3,4,29]. The fact that CBD influenced components of the ECS in the two hypertension models in an opposite manner suggests that a direct antioxidant effect is more likely than an indirect ECS-based mechanism. Finally, although the antioxidant effect of CBD was not associated with an antihypertensive effect it would be interesting to examine the influence of CBD on other cardiovascular parameters, e.g., arrhythmia risk, in future studies. Thus, oxidative stress was shown to be involved in cardiac electrical and structural remodelling [69] and in the pathophysiology of atrial fibrillation [70]. The use of ECG predictors of arrhythmia risk would be a benefit in such studies [69,71].

4. Materials and Methods

4.1. Animals

All procedures and experimental protocols were performed in accordance with the European Directive (2010/63/EU) and with the approval of the local Animal Ethics Committee in Olsztyn (Poland) (Approval code: 80/2017; approval date: 28 November 2017). Rats were obtained from the Center for Experimental Medicine of the Medical University of Białystok (Poland). They had free access to chow and water and were kept under a 12:12 h light-dark cycle. Experiments were performed on male rats with spontaneous (SHR) and secondary DOCA-salt hypertension.

4.2. Experimental Groups and Protocol

DOCA-salt hypertension was induced in Wistar rats. After unilateral nephrectomy of 5-6 week-old animals and one week of recovery, deoxycorticosterone acetate (DOCA) was injected s.c. (25 mg/kg in 0.4 mL DMF/kg) twice a week for 28 days. During DOCA administration, drinking water was replaced with 1% saline water. Control group for DOCA were sham-operated (unilaterally nephrectomised) (SHAM) Wistar rats. They received s.c. DMF (DOCA vehicle) twice weekly for 4 weeks and tap water for drinking.

Animals were randomly divided into experimental groups: (1) hypertensive DOCA rats, (2) respective normotensive control SHAM rats, (3) hypertensive SHR rats and (4) respective normotensive WKY rats. All animal groups were age-matched at the beginning of CBD treatment (8-9 weeks old). The body weight of the rats is shown in Table 1.

4.3. Chronic CBD Administration

One part of every hypertensive and normotensive group were injected i.p. with CBD (10 mg/kg) every 24 h for 14 days. The other part received CBD vehicle (ethanol, Tween 80, 0.9% NaCl—3:1:16; 1 mL/kg).

4.4. Determination of Cardiovascular Parameters in Conscious Rats

Systolic blood pressure (SBP) and heart rate (HR) were measured using non-invasive tail-cuff method with Non-Invasive Blood Pressure Controller (ADInstruments, Sydney, Australia) before first dose of CBD or its vehicle and after 7 and 14 days of experiment (24 h after last dose of compounds).

SBP, diastolic blood pressure (DBP) and HR were also measured telemetrically in SHR and WKY rats, as described previously [15]. Briefly, after pentobarbitone sodium (300 μmol/kg; i.e., ~70 mg/kg; i.p.) anesthesia, telemetry transmitters (HD-S10, Data Sciences International, Saint Paul, MN, USA) were implanted into the femoral artery. Rats were allowed to recover for 1 week before measurements.

4.5. Tissue Preparation for Biochemical Examinations

Twenty-four hours after the last dose of CBD or its vehicle rats were anesthetized with pentobarbitone sodium (300 μmoL/kg; i.p.) to collect blood and heart. Blood samples were obtained by left ventricle puncture and collected into EDTA tubes. Plasma separation from whole blood was carried out by centrifugation at 2000× *g* for 5 min.

Hearts were perfused with 0.9% saline and cut lengthwise into two halves with equal size and quality. The first part of the tissue was snap-frozen with liquid nitrogen and stored at −80 °C. The second part was homogenized. 10% homogenates in 0.9% saline were centrifuged at 20000× *g* for 15 min at 4 °C.

4.6. Biochemical Studies

4.6.1. Determination of Endocannabinoids

Anandamide (AEA), 2-arachidonoylglycerol (2-AG), palmitoyl ethanolamide (PEA), linolenoyl ethanolamide (LEA), oleoyl ethanolamide (OEA), stearoyl ethanolamide (SEA), docosahexaenoyl ethanolamide (DHEA), palmitoleoyl ethanolamide (POEA), homo-γ-linolenyl ethanolamide (HEA), docosatetraenoyl ethanolamide (DEA) and dihomo-γ-linolenoyl ethanolamide (DGLEA) were determined using modified ultrahigh performance liquid chromatography-tandem mass spectrometry (UPL-CMS/MS) by the Lam method [72]. Octadeuterated endocannabinoids: AEA-d_8, 2-AG-d_8 and OEA-d_4 [73] as internal standards were added into the tissue lysates and all cannabinoids were isolated using a solid phase extraction (OASIS HLB 3cc). UPLC–MS/MS analysis was carried out using an Nexera X2 Shimadzu UPLC system with a Zorbax Extend C18 column (2.1 mm× 150 mm, 1.8 mm, Agilent, Santa Clara, CA, USA) and interfaced with a Shimadzu 8060 triple quadrupole mass spectrometer with electrospray ionization source (ESI). The samples were analyzed in positive-ion mode using multiple reaction monitoring (MRM). Transitions of the precursor to the product ion was as follows: m/z 348.3→62.15 for AEA, m/z 379.3→287.25 for 2-AG, m/z 300.3→62.00 for PEA, m/z 324.0→62.00 for LEA, m/z 326.0→62.00 for OEA, m/z 328.0→62.00 for SEA, m/z 372.0→62.00 for DHEA, m/z 298.0→62.00 for POEA, m/z 314.5→62.00 for HEA, m/z 376.0→62.00 for DEA and m/z 350.0→62.00 for DGLEA.

4.6.2. Determination of FAAH and MAGL Activity

Fatty acid amide hydrolase (FAAH) (EC.3.5.1.99) activity was measured in the homogenate of heart tissue prepared in 20 mM Tris, containing 10% glycerol, 150 mM NaCl, and 1% Triton X-100, pH 7.8 at 4 °C. Following centrifugation (1000× *g*), 20 µL of the supernatant was added to 175 µL of reaction buffer (125 mM Tris, pH 9.0, and 1 mM EDTA) and 17 µM of FAAH substrate, decanoyl m-nitroaniline. Formation of m-nitroaniline (m-NA) was determined at 410 nM [74]. Specific enzyme activity was expressed in nmoles of m-NA/min/mg protein.

Monoacylglycerol lipase (MAGL) (EC.3.1.1.23) activity was measured in the homogenate of heart tissue prepared in 20 mM Tris, 320 mM sucrose and 1mM EDTA, pH 8.0. Heart supernatant was obtained from the soluble fraction after spinning the homogenate at 1000× *g* for 15 min. A reaction mixture containing heart supernatants, 10 mM Tris, 1 mM EDTA pH 7.2 was pre-incubated at 4 °C for 15 min. After addition of arachidonoyl-1-thio-glycerol (A-1-TG), the mixture was incubated at 37 °C for 5 min and after refrigerating to room temperature, 1 mM DTNB was added. After 3 min, the formation of TNB was determined at 412 nm. Specific enzyme activity was expressed in nmoles of TNB/min/mg of protein [75].

4.6.3. Western Blot Analysis

A routine Western blotting procedure was used to examine protein expression, as has been described previously [67], except using stain-free technology [76]. Briefly, samples from the left ventricles were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing a cocktail of protease and phosphatase inhibitors. In addition, protein concentration was measured using the bicinchoninic acid method (BCA) with bovine serum albumin (BSA) as a standard. Subsequently, homogenates were reconstituted in Laemmli buffer. The same amounts of protein (30 µg) were loaded on Criterion™ TGX Stain-Free Precast Gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred onto PVDF (polyvinylidene difluoride) membranes using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) and stain-free blot image was taken (ChemiDoc XRS; Bio-Rad, Hercules, CA, USA) for total protein measurement in each sample lane. Next, the membranes were incubated overnight at 4 °C with the corresponding primary antibodies in appropriate dilutions: CB_1 (1:500), CB_2 (1:500), GPR18 (1:5000), GPR55 (1:1000) and TRPV1 (1:500). Thereafter, PVDF membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA). After adding a suitable

substrate for horseradish peroxidase protein bands were detected using a ChemiDoc visualization system XRS (Bio-Rad, Hercules, CA, USA). Thereafter, Western blots were quantified densitometrically with Image Laboratory Software Version 6.0.1 (Bio-Rad, Hercules, CA, USA). The expression of selected target proteins was quantified using stain-free gels and total protein normalization method (Bio-Rad, Hercules, CA, USA).

4.6.4. Determination of Antioxidant Enzyme Activity

Catalase (CAT—EC.1.11.1.9) activity was measured in the homogenate of heart tissue by a spectrophotometric analysis (at 240 nm) of the rate of hydrogen peroxide decomposition, using a method published previously [77]. One unit of CAT is defined as the amount of the enzyme necessary to catalyze the decomposition of 1 μmoL of hydrogen peroxide to water and oxygen within 1 min.

Glutathione reductase (GSR—EC.1.6.4.2) activity was measured according to the method of Mize and Langdon [78] by monitoring the oxidation of NADPH at 340 nm at a pH 7.4. One unit of GSR oxidized 1 μmol of NADPH/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 assessed spectrophotometrically using the method of Paglia and Valentine [79]. GPx activity was assayed by measuring the conversion of NADPH to $NADP^+$. One unit of GPx activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmoL NADPH/min at 25 °C and pH 7.4. Specific enzyme activity was expressed in units per mg of protein.

Superoxide dismutase (SOD—EC.1.15.1.1) activity was measured according the method by Sykes et al. [80]. The oxidation of epinephrine was performed in terms of the production of adrenochrome, which has a maximal absorption at 480 nm. One unit of SOD is defined as the amount of the enzyme that inhibits the rate of autoxidation of epinephrine by 50%.

4.6.5. Determination of Non-Enzymatic Antioxidant Level

Vitamin E and A were detected in the samples using high-performance liquid chromatography (HPLC) [81]. Extraction of vitamins was carried out using hexane. After removal, drying and dilution with ethanol, the hexane phase (50 μL) was injected on the column. UV detection at 294 nm for vitamin E and 298 nm for vitamin A were applied. The flow rate was 1 mL/min of methanol and water (95:5).

Glutathione (GSH) and glutathione disulfide (GSSG) were quantified using the capillary electrophoresis (CE) method of Maeso et al. [82]. Samples were sonificated in the Eppendorf tubes with 2 mL of a mixture containing ACN/H_2O (62.5:37.5, *v*/*v*) and centrifuged at 29,620× *g* for 10 min. The supernatant was immediately measured by CE. The separation was performed on a capillary with 47 cm total length (40 cm effective length) and 50 μm ID and was operated at 27 kV with UV detection at 200 ± 10 nm.

4.6.6. Determination of Protein Modifications

Protein oxidative modifications (carbonyl groups; CO groups) were determined according to the method published previously [83]. Carbonyl content was computed from peak absorption (370 nm) using 2,4-dinitrophenylhydrazine as a reagent.

4.6.7. Determination of Lipid Modifications

Malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) were measured by GC/MSMS, as the O-PFBoxime-TMS derivatives, using modified method of Luo et al. [84]. Benzaldehyde-D6 as an internal standard was added to the tissue lysates and aldehydes were derivatized by the addition of O-(2,3,4,5,6-pentafluorobenzyl) hydroxyamine hydrochloride (0.05 M in PIPES buffer, 200 μL; incubation for 60 min at room temperature). Subsequently, 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. A 1 µL aliquot was injected on the column. Derivatized aldehydes were analyzed using a 7890A GC—7000 quadrupole MS/MS (Agilent Technologies, Palo Alto, CA) equipped with a HP-5 ms capillary column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m length). Derivatized aldehydes were detected in the selected ion monitoring (SIM) mode. The ions used for MDA/4-HNE/4HHE-PFB-TMS identification were m/z 204.0 and 178.0 for MDA; m/z 333.0 and 181.0 for 4-HNE; and 352.0 and 226.0 for 4-HHE respectively and m/z 307.0 for IS (benzaldehyde-D6) derivatives.

4.6.8. Determination of Fatty Acids

The concentration of the fatty acids AA, DHA and LA was determined by gas chromatography [85]. Lipid components were isolated from tissue lysates by extraction with chloroform/methanol mixture (2:1, v/v). Using TLC, total phospholipids were separated with the mobile phase heptane—diisopropyl ether—acetic acid (60:40:3, *v*/*v*/*v*). All lipid fractions were transmethylated to fatty acid methyl esters (FAMEs) with boron trifluoride in methanol reagent under nitrogen atmosphere without previous separation from the layer. The FAMEs were quantified by gas chromatography with a flame ionization detector. Separation of FAME was carried out on a capillary column coated with Varian CP-Sil88 stationary phase and analyzed by gas chromatography with a flame ionization detector (FID) on a Clarus 500 Gas Chromatograph (Perkin Elmer, Waltham, MA, USA).

4.7. Statistical Analysis

The results are expressed as median values and interquartile range. Cardiovascular parameters were obtained from WKY and SHR rats in which blood pressure was recorded telemetrically ($n = 4$). Rats, in which blood pressure was determined by the tail-cuff method, served both for the registration of cardiovascular and biochemical parameters (WKY, SHR, SHAM, DOCA-salt). At the beginning, each group consisted of 7 rats. However, the final n was 5-7 because of (1) the death of one rat (DOCA + CBD) and/or (2) the exclusion of outliers (values deviating from the mean by more than plus/minus three standard deviations). All data were subjected to the Kolmogorov–Smirnov test to assess the distribution of values. If the data were normally distributed, parametric tests were done (paired Student's t-test for comparison within group and one-way ANOVA with Bonferroni's multiple comparison test for multiple groups). Data subjected to ANOVA were followed by Bonferroni's post hoc tests only when the F value attained $p < 0.05$ and there was no significant inhomogeneity of variances. If the data were not normally distributed, a non-parametric test was performed (Wilcoxon test for comparison within group and Kruskal–Wallis test with Dunn's post hoc test to compare multiple groups). Dunn's post hoc test was only used when the Kruskal–Wallis test yielded a significant result ($p < 0.05$). A statistical analysis was performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA).

4.8. 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), 11-deoxycorticosterone acetate (DOCA) (D7000), N,N-dimethylformamide (DMF) (9227056), chloro-2,4-dinitro benzene (CDNB) (237329), butylated hydroxytoluene (BHT) (W218405) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (D8130) from Sigma-Aldrich, Munich, Germany; pentobarbital sodium (5909991290153) from Biowet, Puławy, Poland; anandamide-d8 (AEA-d8) (390050), 2-arachidonoyl glycerol-d_8 (2-AG-d_8) (362160), oleoyl ethanolamide-d_4 (OEA-d_4) (9000552), decanoyl m-nitroaniline (m-NA) (90349), arachidonoyl-1-thio-glycerol (A-1-TG) (10007904) from Cayman Chemical Company, Ann Arbor, MI, USA; CB_1 antibody (ab23703), CB_2 antibody (ab3561), GPR18 antibody (ab174835), GPR55 antibody (ab203663) from Abcam, Cambridge, UK; TRPV1 antibody (bs-1931R) from Bioss Antibodies, Woburn, MA, USA; Clarity Western ECL Substrate (1705060) from Bio-Rad, Hercules, CA, USA.

5. Conclusions

Chronic CBD administration (10 mg/kg once a day for two weeks) does not modify BP and HR in a model of primary (SHR) and secondary (DOCA-salt) hypertension and in their respective normotensive controls in spite of the reduction of cardiac and plasma oxidative stress. Whether, besides its direct effect, CBD also possesses an indirect anti-oxidant effect that is based on the endocannabinoid system is questionable. Thus, CBD had opposite effects on numerous components of the endocannabinoid system in both hypertension models. The unexpected CBD-related increase in lipid peroxidation in normotensive controls deserves further investigation and may lead to untoward effects if CBD is used for therapeutical purposes listed in the Introduction. Provided that our data on animals can be transferred to humans, CBD will not lead to an unexpected fall in blood pressure in patients.

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Abbreviations

2-AG	2-arachidonoylglycerol
4-HHE	4-hydroxyhexenal
4-HNE	4-hydroxynonenal
AA	arachidonic acid
ACN	acetonitrile
AEA	anandamide
A-1-TG	arachidonoyl-1-thio-glycerol
ANOVA	analysis of variance
BCA	bicinchoninic acid
BP	blood pressure
BSA	bovine serum albumin
CAT	catalase
CBD	cannabidiol
CE	capillary electrophoresis
CO	carbonyl groups
DBP	diastolic blood pressure
DEA	docosatetraenoyl ethanolamide
DGLEA	dihomo-γ-linolenoyl ethanolamide
DHA	docosahexaenoic acid
DHEA	docosahexaenoyl ethanolamide
DMF	dimethylformamide
DOCA	deoxycorticosterone acetate *or* DOCA-salt hypertensive rats
DOCA-salt	DOCA-based method to generate hypertension in rats *or* DOCA-salt hypertensive rats
DTNB	5,5′-dithiobis-2-dinitrobenzoic acid
ECG	electrocardiography
ECS	endocannabinoid system
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization source
FAAH	fatty acid amide hydrolase

FAME	fatty acid methyl esters
FFA	free fatty acids
FID	flame ionization detector
GC	gas chromatography
GPR	G-protein coupled receptor
GPx	glutathione peroxidase
GSH	glutathione
GSR	glutathione-disulfide reductase
GSSG	glutathione disulfide
HEA	homo-γ-linolenyl ethanolamide
HPLC	high-performance liquid chromatography
HR	heart rate
ID	internal diameter
i.p.	intraperitoneal injection
IS	internal standard
LA	linoleic acid
LEA	linolenoyl ethanolamide
MAGL	monoacylglycerol lipase
MDA	malondialdehyde
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
m-NA	m-nitroaniline
NADA	N-arachidonoyl dopamine
NADPH	nicotinamide adenine dinucleotide phosphate
OEA	oleoyl ethanolamide
O-PFBoxime	O-(2,3,4,5,6-pentafluorobenzyl) oxime
PEA	palmitoyl ethanolamide
PH	phospholipids
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
POEA	palmitoleoyl ethanolamide
PPR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
RAAS	renin-angiotensin-aldosterone system
RIPA	radioimmunoprecipitation assay
SBP	systolic blood pressure
SEA	stearoyl ethanolamide
SEM	standard error of the mean
SHAM	sham-operated rats
SHR	spontaneously hypertensive rats
SIM	selected ion monitoring mode
SOD	superoxide dismutase
s.c.	subcutaneous injection
THC	Δ^9-tetrahydrocannabinol
TLC	thin-layer chromatography
TNB	5-thio-2-nitrobenzoic acid
TRPV	transient receptor potential vanilloid
UPLC	ultrahigh performance liquid chromatography
WKY	Wistar-Kyoto rats

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Chapter 11.

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Effects of the peripheral CB_1 receptor antagonist JD5037 in mono— and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension

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Pulmonary hypertension (PH) is a disease leading to increased pressure in the pulmonary artery and right heart failure. The adenosine monophosphate-activated protein kinase (AMPK) activator, metformin, has a protective effect against PH. CB_1 receptor blockade reduces the number of pathological alterations in experimental lung fibrosis. The current study evaluates the effect of the peripheral cannabinoid CB_1 receptor antagonist JD5037 in mono- and polytherapy with metformin in rat monocrotaline-induced mild PH. Animals received metformin (100 mg/kg), JD5037 (3 mg/kg), or a combination of both once daily for 21 days. Monocrotaline (60 mg/kg) increased right ventricular (RV) systolic pressure (RVSP), led to RV and lung hypertrophy and remodeling, and decreased oxygen saturation. Metformin partially restored the monocrotaline-induced effects, i.e., decreased RVSP, increased oxygen saturation, and counteracted cardiac fibrotic, hypertrophic, and inflammatory changes. JD5037 modified parameters related to inflammation and/or fibrosis. Only polytherapy with metformin and JD5037 improved Fulton's index and coronary artery hypertrophy and tended to be more effective than monotherapy against alterations in RVSP, oxygen saturation and coronary artery tunica media vacuolization. In conclusion, monotherapy with JD5037 does not markedly influence the PH-related changes. However, polytherapy with metformin tends to be more efficient than any of these compounds alone.

KEYWORDS

AMP-activated protein kinase, cannabinoid 1 receptor, JD5037, metformin, monocrotaline, polytherapy, pulmonary arterial hypertension

Introduction

Pulmonary hypertension (PH) is a rare disease characterized by increased pulmonary arterial pressure over 25 mmHg at rest, which leads to right heart failure and premature death. Among five classified etiological types of PH, pulmonary arterial hypertension (PAH) is the least common but most widely studied one. Its pathophysiology is mainly based on the remodeling of the pulmonary vascular bed with high pulmonary vascular resistance (Simonneau et al., 2019; Mandras et al., 2020; Levine, 2021; Sommer et al., 2021; Zhao et al., 2021). Despite its low incidence and prevalence (ca. 5.8 and 51 per million, respectively) (Leber et al., 2021), PAH is considered a significant issue due to its high mortality (5-years survival rate from 68% in patients with low risk to 23% in the high-risk group) (Hoeper et al., 2017). Currently available pharmacological treatment options, although significantly improving survival statistics (Levine, 2021; Sommer et al., 2021), are not able to cure PAH. Therefore, new strategies that cover more than vasodilation are required (Zhang et al., 2020; Sommer et al., 2021; Zolty, 2021). Here, activation of 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) and blockade of peripheral cannabinoid type 1 (CB_1) receptors will be considered.

AMPK is a sensor of the cellular energy status that senses low cell adenosine triphosphate (ATP) concentration (e.g. during exercise, oxidative stress and hypoxia) (Rodríguez et al., 2021). AMPK is being considered a possible target for PAH treatment, since the endothelial AMPK is downregulated in pulmonary arteries of patients with PAH and knockout of AMPK in mice may accelerate PH progression (Omura et al., 2016). Indeed, AMPK activation has beneficial effects on PAH (Zhao et al., 2021; Zolty, 2021; Flores et al., 2022). E.g., metformin, which is not only important for type 2 diabetes mellitus therapy (Lv and Guo, 2020) but also represents the canonical AMPK activator, decreased proliferation of pulmonary artery smooth muscle cells derived from PAH patients (Dean et al., 2016) and rats exposed to endothelin-1 (Wu et al., 2014) or galectin-3 (Zhang et al., 2021). Moreover, it improved the carbachol-induced relaxation and reduced the phenylephrine-induced contraction of pulmonary arteries isolated from rats with PH elicited by hypoxia (Agard et al., 2009; Deng et al., 2020). In chronic experiments, metformin attenuated PH in rats or mice induced by monocrotaline (MCT) (Agard et al., 2009; Li et al., 2016; Zhai et al., 2018; Yoshida et al., 2020; Sun et al., 2022), hypoxia (Agard et al., 2009; Omura et al., 2016; Liu Y. et al., 2019) and sugen/hypoxia (Dean et al., 2016; Zhang et al., 2018). Moreover, the results of the first of two (Zhao et al., 2021) clinical studies evaluating metformin in PAH have shown that the compound may improve right ventricle (RV) function and reverse some negative metabolic changes in the course of PAH (Brittain et al., 2020). However, other studies suggest that AMPK might facilitate hypoxic pulmonary vasoconstriction (Evans and Hardie, 2020). Thus, further research determining the role of AMPK in PH is still needed (Deng et al., 2020; Zhao et al., 2021).

CB_1 receptors are part of the endocannabinoid system. Their high abundance in the brain is responsible for the psychoactive effect of Δ^9-tetrahydrocannabinol, but thanks to its distribution in almost all body tissues, CB_1 receptors can modulate many different functions (Fowler, 2021). Peripheral overactivity of CB_1 receptors induces cardiac, pulmonary, liver and kidney fibrosis and promotes inflammation and/or oxidative stress (Puhl, 2020; Kicman et al., 2021), and therefore their blockade could become a potential therapeutic strategy (Cinar et al., 2020). In addition, CB_1 receptor expression is increased in the lungs from patients with idiopathic pulmonary fibrosis, which was connected with marked alveolar interstitial collagen deposition (Cinar et al., 2017), and in fibrotic lungs of patients with Hermansky-Pudlak syndrome (Cinar et al., 2021). It has been proved, that genetic deletion of CB_1 receptors or chronic administration of their peripheral antagonists (AM6545 or JD5037) mitigates inflammation and fibrosis and increases animal survival in murine pulmonary fibrosis induced by radiation (Bronova et al., 2015) or bleomycin (Cinar et al., 2017), in experimental liver fibrosis (Tan et al., 2020) and experimental diabetic nephropathy (Barutta et al., 2018).

The gold standard for PAH treatment nowadays is an early combined therapy with compounds that affect different pharmacological targets (Klinger et al., 2019; Mandras et al., 2021; Mayeux et al., 2021; Sommer et al., 2021; Tettey et al., 2021). This kind of therapeutic procedure is superior to therapies with the agents alone as suggested by several meta-analyses based on clinical studies (Sommer et al., 2021) and is recommended by expert guidelines (Klinger et al., 2019). Moreover, PAH is a multi-factorial disease, and the vasoconstriction of pulmonary arteries as the main target of current treatments for PAH appears insufficient. Searching for new targets and treatment strategies not involving pulmonary vasodilation, we studied the influence of separate and combined administration of the peripheral CB_1 receptor antagonist JD5037 and the AMPK activator metformin on the MCT-induced PH in rats.

TABLE 1 Influence of metformin (MET), JD5037 (JD) and their combination (MET + JD) on physiological parameters of monocrotaline-induced pulmonary hypertensive (PH) rats and their normotensive controls (CTR).

Parameter \ Group		CTR + veh	CTR + MET	CTR + JD	CTR + MET + JD	PH + veh	PH + MET	PH + JD	PH + MET + JD
	n	18–20	9–10	8–10	9–10	17–20	9–10	8–10	8–10
Body weight (g)	day 0	313 ± 3	313 ± 6	315 ± 4	316 ± 4	313 ± 2	313 ± 6	316 ± 6	315 ± 4
	day 22	365 ± 5$^{\$\$\$}$	363 ± 8$^{\$\$\$}$	357 ± 6$^{\$\$\$}$	354 ± 6$^{\$\$\$}$	347 ± 4$^{\$\$\$,\ *}$	342 ± 8$^{\$\$\$}$	345 ± 6$^{\$\$\$}$	347 ± 4$^{\$\$\$}$
SBP (mmHg)	day 1	130 ± 3	122 ± 4	122 ± 4	132 ± 6	133 ± 4	137 ± 5	135 ± 10	137 ± 6
	day 22	131 ± 4	131 ± 7	132 ± 9	134 ± 6	131 ± 4	125 ± 2	125 ± 7	129 ± 6
HR	by pulse oximeter	286 ± 2	267 ± 6	283 ± 5	316 ± 9**	293 ± 5	288 ± 10	295 ± 7	296 ± 11
(beats/min)	by catheter	267 ± 6	255 ± 3	249 ± 5	275 ± 6	264 ± 5	261 ± 6	259 ± 4	253 ± 7
dP/dt_{max} (mmHg/s)		1487 ± 45	1482 ± 44	1588 ± 59	1477 ± 51	1804 ± 46***	1730 ± 91	1703 ± 53	1645 ± 68
dP/dt_{min} (mmHg/s)		-1054 ± 30	-1076 ± 54	-1089 ± 55	-1080 ± 35	-1400 ± 56***	-1240 ± 99	-1270 ± 61	-1180 ± 72
Rectal temperature (°C)		35.7 ± 0.2	35.7 ± 0.2	35.6 ± 0.2	36.4 ± 0.3	36.1 ± 0.2	36.2 ± 0.3	35.7 ± 0.3	35.9 ± 0.2
Heart weight/BW (mg/g)		2.83 ± 0.04	2.80 ± 0.04	2.79 ± 0.08	2.79 ± 0.05	2.89 ± 0.06	2.87 ± 0.07	2.91 ± 0.06	2.86 ± 0.07
RA weight/BW (mg/g)		0.106 ± 0.004	0.103 ± 0.004	0.100 ± 0.006	0.118 ± 0.011	0.115 ± 0.005	0.109 ± 0.009	0.117 ± 0.008	0.110 ± 0.010
LA weight/BW (mg/g)		0.069 ± 0.003	0.079 ± 0.006	0.081 ± 0.003	0.078 ± 0.003	0.069 ± 0.003	0.072 ± 0.003	0.070 ± 0.002	0.074 ± 0.003
RV weight/BW (mg/g)		0.448 ± 0.008	0.444 ± 0.014	0.457 ± 0.013	0.462 ± 0.016	0.491 ± 0.012	0.498 ± 0.022	0.510 ± 0.013	0.460 ± 0.016
LV + S weight/BW (mg/g)		1.71 ± 0.02	1.67 ± 0.02	1.70 ± 0.04	1.71 ± 0.03	1.68 ± 0.03	1.73 ± 0.02	1.69 ± 0.04	1.70 ± 0.03
Kidney weight/BW (mg/g)		3.51 ± 0.07	3.55 ± 0.05	3.52 ± 0.06	3.52 ± 0.07	3.59 ± 0.07	3.68 ± 0.06	3.69 ± 0.08	3.61 ± 0.07
Blood glucose (mg/dl)		128 ± 2	125 ± 2	129 ± 4	141 ± 6*	125 ± 3	128 ± 4	132 ± 4	137 ± 7

MET (100 mg/kg), JD5037 (3 mg/kg) or their combination were administered by oral gavage once daily for 21 days (controls received vehicles instead). Measurement of SBP, in conscious and of HR, $dP/dt_{max/min}$ and rectal temperature in anaesthetized animals. Parameters were determined 24 h after the last injection, i.e., on day 22; body weight and SBP, were also determined on days 0 and 1, respectively. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different from CTR + veh, $^{\$\$\$}p < 0.001$ significantly different from day 0. *n*, the number of rats per group; in CTR + veh and PH + veh, *n* was double as high because both groups, which did not differ in their results, were combined.
veh–vehicle; SBP, systolic blood pressure; HR, heart rate; dP/dt_{max}, dP/dt_{min}–rate of rise/decrease of right ventricular pressure; BW, body weight; RA, right atrium; LA, left atrium; RV, right ventricle; LV + S–left ventricle + septum.

Materials and methods

Animals

All procedures and experimental protocols were performed in accordance with the European Directive (2010/63/EU) and with the approval of the local Animal Ethics Committee in Olsztyn (Poland) (approval codes 74/2020, 9/WNP/WDO/2021 and 39/WNP/2021). Rats were obtained from the Centre for Experimental Medicine of the Medical University of Bialystok (Poland). They had free access to chow and water and were kept under a 12:12 h light-dark cycle and constant temperature (21 ± 2°C) and humidity (55 ± 5%).

Protocol and experimental groups

On day 0, male Wistar rats were given a single subcutaneous (s.c.) injection of monocrotaline (MCT) (60 mg/kg in a volume of 3 ml/kg) to induce pulmonary hypertension (PH) (Sadowska et al., 2020). The studied compounds were administered in a preventive regimen. Controls (CTR) received a s.c. injection of an equal volume of 0.9% NaCl. From day 1 onward, metformin (100 mg/kg; MET), JD5037 (3 mg/kg; JD), the combination of metformin and JD5037 or their vehicles (veh; *metformin*: 0.9% NaCl, 5 ml/kg; *JD5037*: DMSO, Tween 80 and 0.9% NaCl 4:1:95, 5 ml/kg) were administered to CTR and PH rats by oral gavage every 24 h for 21 days.

There were 8 groups of animals: 1) CTR + veh, 2) CTR + MET, 3) CTR + JD, 4) CTR + MET + JD, 5) PH + veh, 6) PH + MET, 7) PH + JD and 8) PH + MET + JD. Originally, there were even 10 groups. However, the groups "CTR + veh for MET" and "CTR + veh for JD" and the groups "PH + veh for MET" and "PH + veh for JD" were combined since the respective values did not differ significantly (Table 1).

Animals were randomly allocated to the experimental groups and did not differ with respect to weight (see Table 1) and age (9–11 weeks old) at the beginning of the protocol.

Determination of cardiovascular parameters in conscious rats

Systolic blood pressure (SBP) was measured using the non-invasive tail-cuff method with the Non-Invasive Blood Pressure

Controller (ADInstruments, Sydney, Australia) after the administration of MCT or its vehicle before the first dose of metformin and/or JD5037 or their vehicle and after completion of the study (24 h after the last injection).

Determination of blood glucose level

Blood glucose level was measured in blood samples from the lateral tail vein using the Accu-Chek blood glucose meter (Roche Diabetes Care GmbH, Mannheim, Germany).

Determination of right ventricular systolic pressure

After induction of anaesthesia with ketamine and xylazine (i.p., 1 ml of ketamine 100 mg/ml + 100 µl of xylazine 20 mg/ml; 300 µl per 250 g of body weight), a pressure catheter with a sensor for the measurement of the right ventricular systolic pressure (RVSP), HR and the rate of rise/decrease of right ventricular pressure ($dP/dt_{min/max}$) (SPR-320 Mikro-Tip, Millar, Houston, TX, USA) was pushed forward through the right jugular vein and placed in the right ventricle. Data were acquired using LabChart 7.3.7 Pro (ADInstruments, Sydney, Australia).

Determination of blood oxygen saturation

Blood oxygen saturation and heart rate (HR) were measured using a pulse oximeter (MouseSTAT® Jr Rodent Pulse Oximeter and Heart Rate Monitor with Rat Paw Pulse Oximeter Sensor, Kent Scientific Corporation, Torrington, CT, USA) attached to the left front paw of the animal right after anaesthesia and after placing the rat on a heating pad (Bio-Sys-Tech, Białystok, Poland).

Determination of rectal temperature

Rectal temperature was measured using a rectal probe transducer (RDT 100; Bio-Sys-Tech, Białystok, Poland) right after anaesthesia.

Determination of organ weight and hypertrophy indexes

After RVSP determination, the heart and lungs were removed. Next, the right ventricle (RV), left ventricle with septum (LV + S), right (RA) and left (LA) atria, left lung and left kidney were separated and weighed. Right ventricle hypertrophy was expressed in two ways: as Fulton's index, which is RV weight to LV + S weight (RV/LV + S) and as right ventricular hypertrophy index, which is RV weight to body weight of the animal. Lung hypertrophy index was expressed as left lung weight to body weight of the animal.

Histopathology

After separation and weighing, RV and right lung were fixed with 10% buffered formalin. The tissue was paraffin-embedded and cross-sectioned at 5-µm thickness; sections were subjected to hematoxylin and eosin (H&E) staining.

For the quantification of the pulmonary artery vascular wall thickness, the % wall area was calculated from the area of smooth muscle (total area of the vessels - lumen area of the vessels), divided by the total area of vessels (Jiang et al., 2021). Ten vessels per lung were counted. Only arteries with a diameter ranging from 50 to 150 µm were included in the statistical calculations. Mean vessel size was comparable among groups and amounted to approximately 74 µm.

Histopathological evaluations of hearts and lungs were performed by a veterinary pathologist with a specialization in the pathology of laboratory animals. The criteria for histopathological evaluation were based on the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) guidelines (Berridge et al., 2016) developed by ESTP, STP, BSTP and JSTP. Histopathological assessments were made in a system describing the organ, histological structure, pathological change, and the severity of the pathological change on a scale of 0–4.

Western blot analysis

After separation and weighing, the left lung was rinsed with 0.9% saline, drained and snap-frozen with liquid nitrogen and stored at −80°C. After pulverization, samples were homogenized in a protein extraction reagent containing a cocktail of protease inhibitors and centrifuged at 10,000 × g for 10 min at 4°C. In addition, protein concentration was measured using the bicinchoninic acid method (BCA) with bovine serum albumin (BSA) as a standard. Subsequently, homogenates were reconstituted in Laemmli buffer with a reducing agent. The same amounts of protein (30 µg) were loaded on a polyacrylamide gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Next, the membranes were blocked to minimalize non-specific signal and incubated overnight at 4°C with the corresponding primary antibodies in appropriate dilutions: galectin-3 (1: 5,000) and TGF-β1 (1:1000). Thereafter, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. After adding a suitable substrate,

protein bands were detected using a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, United States). Then, Western blots were quantified densitometrically with ImageJ 1.53p software (National Institutes of Health, Bethesda, MD, USA). The expression of selected target proteins was standardized to β-actin expression.

Statistical analysis

Results are expressed as mean values ± standard error of the mean (SEM) or medians with an interquartile range. At the beginning, each of the 10 groups consisted of 10 rats. However, the final n was 6–20 because 1) the groups receiving the solvent for MET and JD5037 were combined (no significant differences between the values), 2) measurement of hemodynamic parameters failed in a few cases and/or 3) outliers (values deviating from the mean by more than plus/minus three standard deviations) were excluded. To obtain an accurate group size (n = 5) in WB analysis and meet the requirement of running all samples on a single gel, normotensive groups receiving the treatment were excluded (no changes observed in preliminary experiments). All data were subjected to the Kolmogorov-Smirnov test to assess the distribution of values. If the data were normally distributed, the (parametric) one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test for multiple groups or paired Student's t-test for comparison within the group was carried out. Data subjected to ANOVA were followed by Bonferroni's post hoc tests only when the F value attained $p < 0.05$ and there was no significant inhomogeneity of variances. Histopathological scoring was performed on the basis of an ordinal scale and for this reason the nonparametric Kruskal-Wallis test with Dunn's post hoc test was used (Gibson-Corley et al., 2013). Statistical analysis was performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, United States).

Drugs

JD5037 (2S)-2-[[[(4S)-5-(4-chlorophenyl)-4-phenyl-3,4-dihydropyrazol-2-yl]-[(4-chlorophenyl)sulfonylamino]methylidene]amino]-3-methylbutanamide (530481) from MedKoo Biosciences, Morrisville, NC, United States; metformin (PA-03–2747-P-25G) from POL-AURA, Różnowo, Poland; monocrotaline (C2401) from Sigma-Aldrich, Burlington, MA, USA; ketamine (5909997022796) from Biowet, Puławy, Poland; xylazine (5909997021911) from Vetoquinol Biowet, Gorzów Wielkopolski, Poland; anti-galectin-3 antibody (ab76245), anti-TGF-β1 antibody (ab179695) and anti-β-actin antibody (ab8227) from Abcam, Cambridge, United Kingdom.

Results

General

As shown in Table 1, the body weight of rats was similar among all groups on day 0 and increased during the subsequent 22 days. No mortality following MCT administration was observed. On day 22, the body weight in the control group (CTR; no MCT, no drugs) was higher by about 5% than in the corresponding PH group. Systolic blood pressure (SBP) measured non-invasively before the first and after the last dose of the studied compounds was comparable in normotensive and PH groups. Although PH did not affect heart rate (HR), combined administration of JD5037 (3 mg/kg) and metformin (100 mg/kg) significantly increased (+ 10%; measured by a pulse oximeter) or tended to increase (catheter) this parameter in the control group. The rates of rise (dP/dt_{max}) and of decrease (dP/dt_{min}) of right ventricular pressure increased (+21%) and became significantly more negative (−33%), respectively, in the PH vs. the control group. There was no influence of PH or therapy on rectal temperature and kidney hypertrophy index. With respect to cardiac hypertrophy indexes, PH only tended to increase the parameter of the right atrium and ventricle. The combined therapy of metformin and JD5037 tended to decrease the right ventricle hypertrophy index and to normalize the PH-induced changes in dP/dt_{max} and dP/dt_{min}. Polytherapy increased blood glucose level in controls (+10%) and tended to do so in animals with PH.

Influence of PH and drug therapies on RVSP, Fulton's index and blood oxygen saturation

As shown in Figure 1, right ventricular systolic pressure (RVSP) and Fulton's index were higher (by 39% and 16%, respectively) and blood oxygen saturation was lower (by 4%) in the PH than in the normotensive control group. Chronic 21-day administration of metformin partially normalized the changes associated with PH, i.e. decreased RVSP by 15% and increased oxygen saturation by 3%. There was no effect of metformin on Fulton's index or of JD5037 on any of the three parameters in the PH group. The combined therapy of metformin and JD5037 tended to have stronger effects on the PH-induced changes than the monotherapies, i.e. decreased RVSP by 18% and increased oxygen saturation by 4%. A statistically significant effect on Fulton's index (decrease by 11%) was observed after combination therapy only.

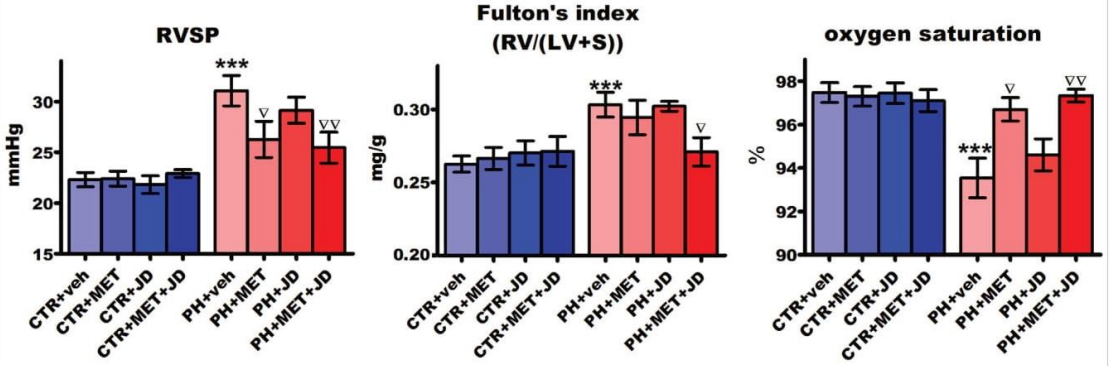

FIGURE 1
Influence of pulmonary hypertension, metformin (MET), JD5037 (JD) and/or their combination on right ventricular systolic pressure (RVSP), Fulton's index (RV/(LV + S)) and oxygen saturation in monocrotaline-induced pulmonary hypertensive (PH) rats and their normotensive controls (CTR). MET (100 mg/kg), JD5037 (3 mg/kg) or their combination were administered by oral gavage once daily for 21 days; controls received vehicles instead. Data are expressed as mean ± SEM; n = 9–20; ***$p < 0.001$ significantly different from CTR + veh; $^{\triangledown}p < 0.05$; $^{\triangledown\triangledown}p < 0.01$ significantly different from PH + veh. RV–right ventricle; LV + S–left ventricle + interventricular septum.

TABLE 2 Influence of metformin (MET), JD5037 (JD) and their combination (MET + JD) on histopathological right ventricular scoring of monocrotaline-induced pulmonary hypertensive (PH) rats and their normotensive controls (CTR).

Anatomical structure/parameter \ Group		CTR+veh	PH+veh	PH+MET	PH+JD	PH+MET+JD
	n	7	6-7	7	7	7
cardiomyocytes	hypertrophy[a]	0 (0;0)	2 (1;2) **	1.5 (0;2)	1.5 (1;2)	1.5 (0;3)
	cytoplasm vacuolization[a]	1 (0;2)	2 (2;2)	0 (0;1)	0 (0;3)	0 (0;1)
	striation visibility decreased[a]	0 (0;0)	1 (1;2)	2 (0;2)	2 (1;2.5)	2 (1;2.5)
	karyomegaly[b]	1 (0;2)	2 (1;2)	1.5 (1;2)	2 (1.5;2)	2 (0;3)
	fragmentation/lysis of muscle fibers[a]	0 (0;0)	1 (0;1)	0 (0;1)	0 (0;1)	0 (0;2)
	cytoplasmic hypereosinophilia[a]	0 (0;0)	2 (2;3) **	1 (0;2)	2 (0;2.5)	1.5 (0;2)
	pyknosis[a]	0 (0;0)	1 (1;1)	0 (0;1)	0 (0;2)	1 (0;2)
myocardium	waviness of muscle fibers[a]	0 (0;0)	2 (2;2) **	1 (0;2)	1 (1;2)	1 (0;2.5)
	hyperplasia of connective tissue[a]	0 (0;1)	2 (2;3) **	0 (0;1) ▽▽	0 (0;0) ▽▽▽	0 (0;1) ▽▽
	increased infiltration of mast cells[c]	1 (1;1)	1 (1;1)	1 (0;2)	0 (0;0) ▽	1 (0;1)
	increased infiltration of mononuclear cells[c]	1 (1;1)	3 (2;3)	1 (0;2)	0 (0;1) ▽▽	0 (0;1) ▽▽
	extravasation[d]	0 (0;0)	1 (0;2) *	1 (1;1)	1 (1;1)	1 (1;2)
coronary arteries	congestion[d]	1 (0;1)	2 (1;2)	1 (1;1)	1 (1;2)	1 (1;2)
	tunica media hypertrophy[a]	0 (0;1)	3 (2.5;3) *	1 (0;2)	1 (0;2)	0 (0;1) ▽▽
	tunica media vacuolization[a]	0 (0;0)	3 (2.25;3) **	0 (0;1) ▽	0 (0;1) ▽	0 (0;0) ▽▽
	increased infiltration of mononuclear cells[a]	0 (0;0)	2 (0.75;2) *	0 (0;0.5)	0 (0;0) ▽	0 (0;0) ▽
pericardium	increased infiltration of plasmacytic/mast cells[c]	0 (0;1)	1 (1;1)	0 (0;0) ▽▽▽	0 (0;0) ▽▽▽	0 (0;0) ▽▽▽

MET (100 mg/kg), JD5037 (3 mg/kg) or their combination were administered by oral gavage once daily for 21 days; controls received vehicles (veh) instead. Data are based on 6-7 rats per group and are expressed as median of scores ranging from 0 to 4 with an interquartile range. *$p < 0.05$, **$p < 0.01$ significantly different from CTR + veh. $^{\triangledown}p < 0.05$, $^{\triangledown\triangledown}p < 0.01$, $^{\triangledown\triangledown\triangledown}p < 0.001$ significantly different from PH + veh. The colors correspond to the median values of the scoring of the group: dark green (0), light green (1), yellow (1.5), orange (2) and red (3).
[a]Scoring scale type B5: 0—no pathological changes; 1—minimal disruptions in architecture/structure, ×40 objective; 2—moderate disruptions in architecture/structure, ×40 objective; 3—minimal/mild disruptions in architecture/structure, ×10 objective; 4—moderate/marked disruptions in architecture/structure, ×10 objective.
[b]Scoring scale type A5: 0—0–5%; 1—6–25%; 2–26–50%; 3–51–75%; 4–76–100%.
[c]Scoring scale type C3: 0—no pathological changes, ×40 objective; 1—up to 2 foci; 2—3-4 foci; 3—5-6 foci; 4—>6 foci.
[d]Scoring scale type B4: 0—no pathological changes; 1—minimal/mild increase of changes, ×40 objective; 2—mild severity of changes, ×10 objective; 3—moderate severity of changes, ×10 objective; 4—moderate/marked severity of changes, ×4 objective.

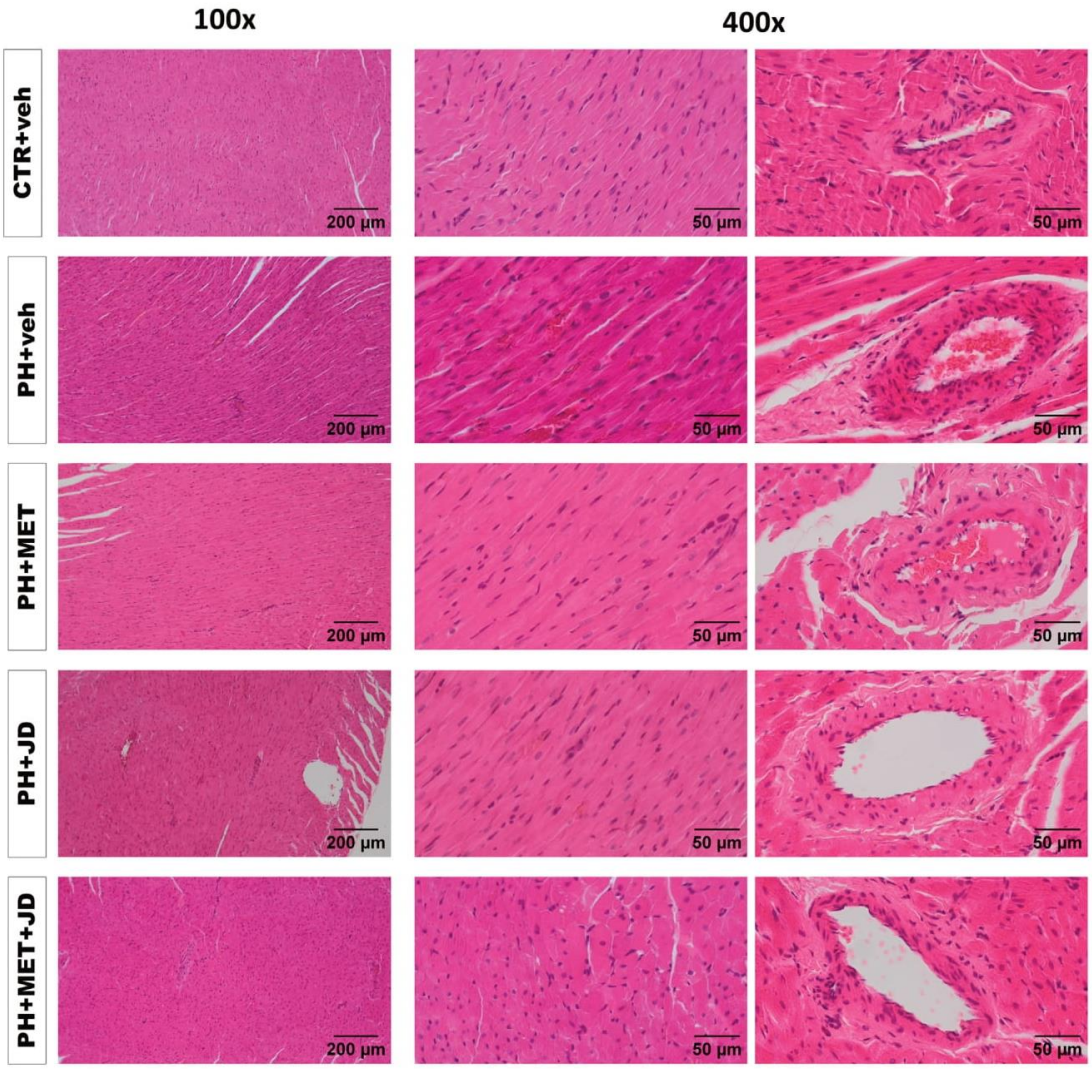

FIGURE 2
Influence of pulmonary hypertension, metformin (MET), JD5037 (JD) and/or their combination on right ventricular remodeling in monocrotaline-induced pulmonary hypertensive (PH) rats and their normotensive controls (CTR). Representative hematoxylin and eosin-stained right ventricle images (100x and 400x magnification); quantitative evaluation is provided in Table 2. MET (100 mg/kg), JD5037 (3 mg/kg) or their combination were administered by oral gavage once daily for 21 days; controls received vehicles (veh) instead.

Influence of PH and drug therapies on the right ventricle

As shown in Table 2 and Figure 2, PH caused changes in right ventricle tissue assessed by histological scoring. Compared to the control group, significant alterations can be observed in cardiomyocytes (hypertrophy and hypereosinophilia of cytoplasm, i.e., an increase in the intensity of eosin staining), myocardium (increased waviness of muscle fibers, hyperplasia of connective tissue and extravasation) and coronary arteries (hypertrophy and vacuolization of tunica media and increased infiltration of mononuclear cells). Tendencies of changes connected with PH vs. control can be found for almost every histopathological parameter assessed. In addition, only tendencies in Masson trichrome collagen staining were observed (Supplementary Table S1). Metformin or

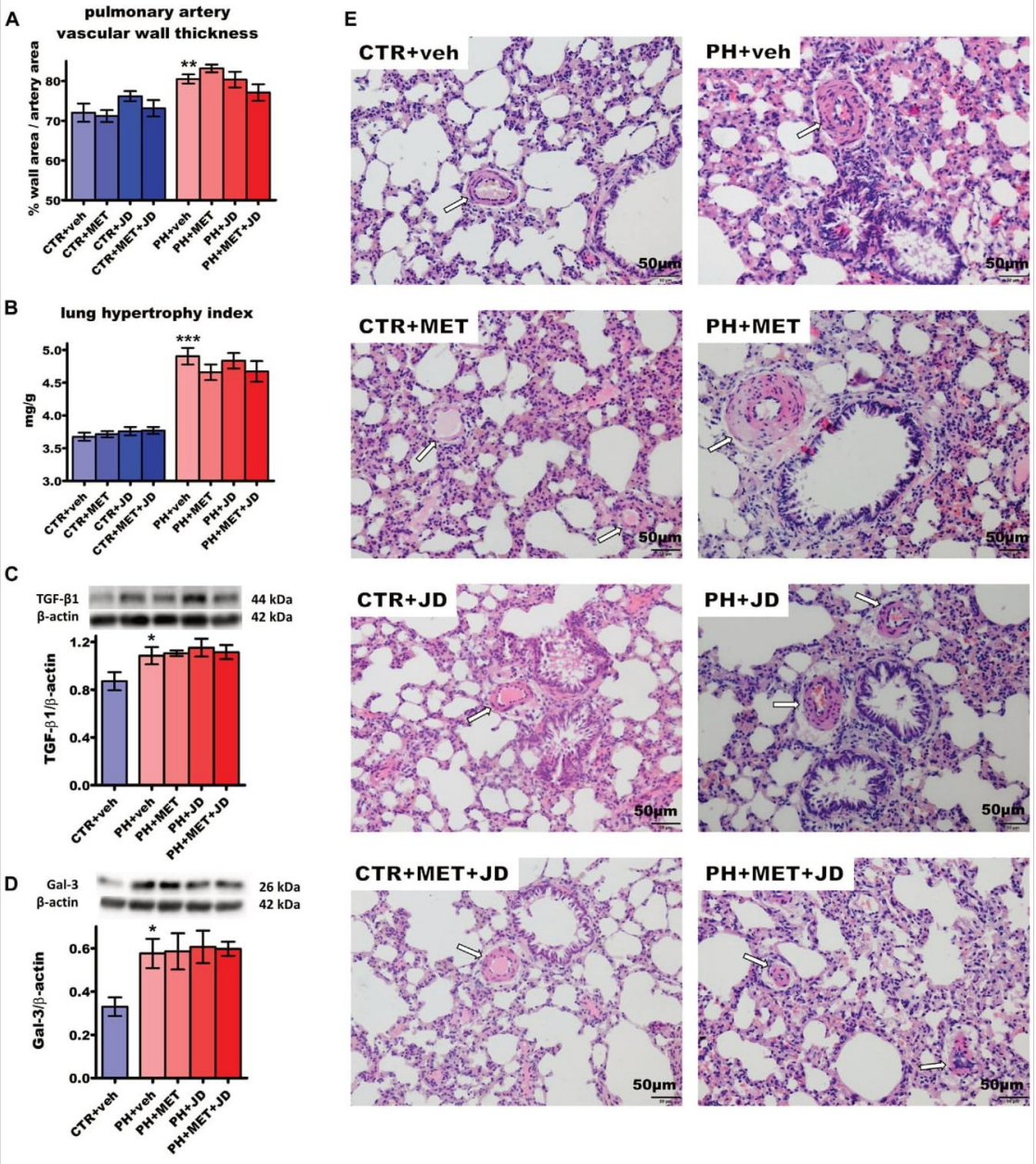

FIGURE 3
Influence of pulmonary hypertension, metformin (MET), JD5037 (JD) and/or their combination on pulmonary remodeling in monocrotaline-induced pulmonary hypertensive (PH) rats and their normotensive controls (CTR). **(A)** pulmonary artery vascular wall thickness, **(B)** lung hypertrophy index (expressed as lung weight to body weight) and expression of **(C)** transforming growth factor β1 (TGF-β1) and **(D)** galectin-3 (Gal-3) determined by Western blot (WB) technique, **(E)** representative hematoxylin and eosin-stained lung images (200x magnification). β-actin served as a loading control in WB. Arrows on images show the location of the vessels. MET (100 mg/kg), JD5037 (3 mg/kg) or their combination were administered by oral gavage once daily for 21 days; controls received vehicles (veh) instead. Data are expressed as mean ± SEM; $n = 9–20$ **(A–B)**, $n = 5$ **(C,D)**; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ significantly different from CTR + veh.

JD5037 administered alone decreased hyperplasia of connective tissue in myocardium, vacuolization of tunica media of coronary arteries and infiltration of plasmacytic and/or mast cells in pericardium, which were or tended to be elevated in PH. JD5037, unlike metformin, decreased mast cell infiltration in myocardium and the infiltration of mononuclear cells in myocardium and coronary arteries. Combined therapy with metformin and JD5037 influenced the same parameters as in monotherapies with similar or better results, except for tunica media hypertrophy of coronary arteries, for which it was the only effective treatment.

Influence of PH and drug therapies on the lungs

As shown in Figures 3A–D, PH increased pulmonary artery vascular wall thickness by 12%, the lung hypertrophy index by 33%, transforming growth factor β1 (TGF-β1) expression in the lung by 25% and galectin-3 expression by 75% compared to the normotensive control. In addition, PH rats showed an increased medial wall thickness, stenosis of arteries, inflammatory cells infiltration and thickening of interalveolar partitions (for representative images, see Figure 3E). However, the degree of muscularization in pulmonary vessels was not increased by PH (Supplementary Table S1). PH-induced alterations were not influenced by metformin, JD5037 or their combination in a statistically significant manner; only tendencies were observed. Thus, metformin tended to diminish the lung hypertrophy index and the combined therapy, in addition, tended to reduce pulmonary artery vascular wall thickness.

Discussion

This study shows for the first time that the peripheral CB_1 receptor antagonist JD5037 has beneficial effects in a monocrotaline protocol of the rat associated with mild pulmonary hypertension. Moreover, the beneficial effects of another protective agent, the AMPK activator metformin, are further increased or only become significant in combination with JD5037.

Methodological considerations

Using MCT, we applied one of the most accepted preclinical rodent models of established PH (also used for the development of PAH-targeted therapies). Rats were preferred since rapid metabolism of MCT occurs in mice (Dignam et al., 2022). A dose of 60 mg/kg is sufficiently high to lead to the pathological features of PH (Bonnet et al., 2017; Dignam et al., 2022; Jama et al., 2022). Metformin was administered at 100 mg/kg for 21 days since this protocol had a beneficial effect on PH induced by hypoxia (i.p., 21 days) (Liu et al., 2019) and sugen/hypoxia (orally, 21 days) (Dean et al., 2016) in rats. JD5037 was applied at 3 mg/kg for 21 days since similar protocols proved effective against liver fibrosis (orally, 2 or 8 weeks) (Tan et al., 2020) or obesity-induced chronic kidney disease (orally, 28 days) (Udi et al., 2020) in rodents.

Induction of pulmonary hypertension

Our study shows that PH is developing during 21 days after MCT injection and leads to changes of cardiac and pulmonary parameters. RVSP, the main determinant of PH development, was elevated by about 40%. The value of the pressure in the right ventricle of about 30 mmHg is not high and allows to classify the resulting hypertension as mild or early-stage PH. Very similar observations have been made in experiments conducted on rats of comparable age from exactly the same source as ours by another group (Hołda et al., 2020a; Hołda et al., 2020b). In addition, analogous values of RVSP were observed previously in rats using the RV catheterization technique and even in some longer-lasting protocols the effect was not further increased (Agard et al., 2009; Dai et al., 2010; Ou et al., 2010; Gubrij et al., 2014; Meghwani et al., 2018; Jin et al., 2019; Cao et al., 2020; Videja et al., 2021; Sun et al., 2022). However, we would like to underline that the pathophysiological basis of early PAH is not sufficiently understood. Very rapid progression of the disease that leads to RV failure induced by MCT (to a lesser extent also by other factors that cause experimental PH) may prevent the development of compensatory mechanisms that normally occur in humans and is being considered as one of the weaknesses of this model. Many effective therapies in animal studies have not been translated into clinical trials, because they do not completely reflect human PAH (Dignam et al., 2022). Moreover, the recently proposed lowering of the diagnostic threshold for pulmonary hypertension brings a new challenge, namely the search for treatments that would be effective in patients with lower pulmonary pressure values. All presently used therapies have been studied in cases with advanced PH and it is unclear if currently available treatment schedules will be helpful for them (Hoeper and Humbert, 2019; Stewart et al., 2020; Sommer et al., 2021). That is why research into PH in its early stages is extremely important to find the right treatment options for a new group of patients.

In our previous study (Sadowska et al., 2020) we had used 5–8 week-old rats but we preferred 9–11 week-old animals in the present one to avoid premature deaths. Kawade et al. (2021) compared 7 and 20-week-old rats and found that MCT led to a much higher survival rate but also induced less severe PH in older compared to younger rats. This observation also translated into our experience and that of another group using animals from exactly the same source as ours (Hołda et al., 2020a); in both

instances, no animal mortality was observed because of the development of mild PAH.

The increase in RVSP was associated with a significant enlargement of RV, expressed as Fulton's index, and a tendency of an increase in RV weight/body weight ratio and heart weight/body weight ratio, like in our previous paper (Sadowska et al., 2020). The PH animals also showed increased rates of rise (dP/dt_{max}) and decrease (dP/dt_{min}) of right ventricular pressure. These data suggest that in our model RV maintains its function by increasing contractile (inotropic) and lusitropic action and is still in a compensatory phase (Vélez-Rendón et al., 2018; Oknińska et al., 2021). In addition, the histopathological results support functional and organ hypertrophic changes in RV. Thus, PH led to cardiomyocyte hypertrophy, cardiomyocyte hypereosinophilia and a wavy arrangement of myocardial fibers, fibrotic and inflammatory modifications (also of the coronary arteries) and tissue damage.

PH also caused pulmonary alterations, such as lung hypertrophy and an increase of pulmonary arterial wall thickness, which had been previously described, among others, also by our group (Sadowska et al., 2020). In the present paper, the PH-induced increase in pulmonary arterial wall thickness cannot be related to a proliferation of vascular smooth muscle cells since PH did not lead to changes in muscularization. The macroscopic findings were reflected by biochemical alterations in lung tissue, i.e. an increase in TGF-β1 and Gal-3 expression. Gal-3 in PH is associated with an impairment of redox balance and induction of inflammation, both of which contribute to vascular fibrosis and remodeling (Fulton et al., 2019; Barman et al., 2021). In addition, Gal-3 interacts with many signaling molecules, including TGF-β1, which induces fibrosis during chronic inflammatory diseases (Weiskirchen et al., 2019), plays a crucial role in the PAH pathogenesis and is a promising target to treat this disease (Sanada et al., 2021; Andre et al., 2022). Both parameters are therefore early predictors of tissue remodeling that have been observed in our model.

To summarize, hemodynamic, histological and biochemical parameters determined in our study 3 weeks after MCT administration are characteristic for mild PH only. The most severe changes (including evident fibrosis) are observed mainly 4 weeks after MCT administration (Xu et al., 2018; Hołda et al., 2020a; Padrez et al., 2022).

Effects of metformin and/or JD5037 on PH

In previous studies (Agard et al., 2009; Li et al., 2016; Zhai et al., 2018; Sun et al., 2019; Yoshida et al., 2020), metformin was proved effective in attenuating the MCT-induced PH-related changes in rats, such as increased RVSP, Fulton's index, pulmonary arterial thickness or lung tissue collagen deposition. In our hands, metformin partially restored the PH-induced RVSP increase and the decreased blood oxygen saturation. Moreover, in the histological part of our study, it attenuated the fibrotic, hypertrophic and inflammatory alterations induced by PH. The lack of a significant influence on macroscopic parameters related to RV and pulmonary vascular hypertrophy may result from the (too high) age of the animals at the time of PH induction and/or the (too short) duration of the experimental protocol, since metformin was applied for 21 days in our hands as opposed to 28–30 days in the publications listed above. The fact that metformin lowers RVSP (in fact, partially prevents it from increasing), but does not affect Fulton's index may come as a surprise. However, it should be noted that RV hypertrophy is not necessarily solely due to an increased afterload. Another reason may be the direct toxic effect of MCT on the heart including right ventricle hypertrophy and myocarditis (Jasińska-Stroschein, 2021; Dignam et al., 2022). Metformin has been used for years as an antidiabetic agent (Lv and Guo, 2020) and is a safe and well-tolerated drug, as also suggested by our experiments on control and MCT-treated rats. Its lack of an effect on blood glucose levels suggests that its beneficial effects on some of the PH-related alterations are not associated with its anti-diabetic properties.

The CB_1 receptor antagonist JD5037 is a quite new compound and has been tested so far in animal models only, in which, like in our own experiments, no serious adverse effects were found (Kale et al., 2019). The fact that the activity of JD5037 is restricted to the periphery is advantageous since CB_1 receptor antagonists penetrating the blood-brain barrier may lead to severe central side effects; for this reason, rimonabant had even to be withdrawn from the market (Cinar et al., 2020). In our hands, chronic administration of JD5037 alone improved or tended to improve some microscopic and biochemical parameters related to inflammation (infiltration of immune cells in myocardium, coronary arteries and pericardium) and/or fibrosis (hyperplasia of myocardial connective tissue and tunica media hypertrophy). These data are not surprising, since CB_1 receptor overstimulation or overactivity leads to cardiac dysfunction, inflammation or oxidative stress (Puhl, 2020) and pulmonary injury with inflammation and fibrosis (Zawatsky et al., 2020; Cinar et al., 2022). In addition, CB_1 receptor blockade was effective as an anti-inflammatory and anti-fibrotic strategy in animal models (Bronova et al., 2015; Cinar et al., 2017; Barutta et al., 2018; Tan et al., 2020). On the other hand, one has to admit that in our PH model JD5037 did not act against several aspects of the MCT-induced PH and, in particular, did not modify the main changes in RVSP, Fulton's index or oxygen saturation.

Combined 21-day therapy with metformin and JD5037 was found to be an effective strategy against the PH-related alterations in RVSP, Fulton's index, oxygen saturation or the histopathological hypertrophic and inflammatory changes in RV tissue. Only the dual treatment decreased Fulton's index and the hypertrophy in the media of coronary arteries and tended to decrease RV/body weight

ratio. In addition, the PH-induced increase in RVSP and the decrease of blood oxygen levels tended to be further improved after polytherapy when compared to monotherapy with metformin. Moreover, the combination therapy tended to decrease pulmonary artery vascular thickness and to normalize dP/dt. An interesting observation is that both mono- and polytherapy do not modify biochemical predictors of lung tissue remodeling (TGFβ-1 and Gal-3). In general, all pulmonary parameters are not or only weakly amenable to therapy.

To summarize, mechanistically, both metformin and JD5037 were effective against hallmarks of mild PH connected with inflammation and remodeling. Furthermore, in both cases myocardial cellular and/or vascular antihypertrophic activity was found. The reason for the superior effect of the combination therapy against most parameters of MCT-induced PH may be that two complementary mechanisms act in parallel. However, a potential additive effect of both compounds is conceivable as well since JD5037 (and other CB_1 receptor inhibitors) may act also via activation of AMPK (Liu et al., 2019). On the other hand, two explanations can be excluded. Thus, the combination therapy increased rather than decreased both blood glucose level and HR although changes were very slight. When given alone, neither the CB_1 receptor antagonist AM251 (Weresa et al., 2019) nor metformin (Zilov et al., 2019) modifies cardiovascular parameters. Metformin is well-known for its glycemia-reducing activity in diabetic individuals (Lv and Guo, 2020) and JD5037 improves glucose metabolism in obese (Tam et al., 2012; Cinar et al., 2014; Knani et al., 2016; Liu et al., 2019) and diabetic (Hinden et al., 2018) mice. In addition, the CB_1 receptor antagonists rimonabant (Christopoulou and Kiortsis, 2011) and taranabant (Kipnes et al., 2010) used in clinical trials of type 2 diabetes reduced the level of glycated hemoglobin in metformin-treated patients. Probably an unknown interaction is responsible for the unexpected increase in glucose and HR and further research is needed to elucidate its mechanism.

Limitations of the study

In the present study, we examined the potential preventive effects of a three-week-long administration of metformin (100 mg/kg), JD5037 (3 mg/kg) or their combination on MCT-induced PH in male rats. Thus, one should keep in mind that other results may be obtained if 1) experimental PH is induced by other stimuli, e.g., sugen plus hypoxia; 2) a therapeutic rather than a preventive paradigm is used; 3) compounds are administered at a higher dose or for a longer time; 4) the end-stage PH is studied (obtained by a longer period of PH development, the use of younger rats and/or another strain, e.g., Sprague-Dawley) and 5) the PH is induced in female animals. Despite the fact that PAH develops predominantly in women (Docherty et al., 2018; Hester et al., 2019), we used male rats since the development of the MCT-induced PH is less pronounced in female animals (Frump et al., 2021).

Conclusion

Chronic 21-day combined administration of metformin (100 mg/kg) and JD5037 (3 mg/kg) attenuated most of the mild PH-induced cardiopulmonary alterations and tended to be more efficient than any of the monotherapies alone. Quite recently third-generation CB_1 receptor antagonists have been synthesized, i.e., compounds combining peripherally restricted CB_1 receptor antagonism with an additional target. AMPK activation is suggested as a possible secondary target for such hybrid molecules (Cinar et al., 2020) and dual compounds combining peripheral CB_1 antagonism and AMPK activation have been presented in an abstract form very recently (Iyer et al., 2022). Our results argue in favour of further studies dedicated to such hybrid compounds. In such studies, the significance of the combined therapy of peripheral CB_1 receptor antagonism plus AMPK activation in pulmonary arterial hypertension, both in its early and end-stage phases, should be taken into consideration. Moreover, one should keep in mind that the third week after the monocrotaline administration is critical for PH development and might result in the induction of both early and end-stage PH.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Local Animal Ethics Committee in Olsztyn (Poland).

Author contributions

PR designed the methodology, developed the model, performed experiments, analyzed the data, created figures and wrote the original draft. AP-B performed experiments and reviewed the original draft. KM, JK, and JD performed experiments. ES reviewed the original draft. BM conceptualized and supervised the project and reviewed the original draft. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.965613/full#supplementary-material

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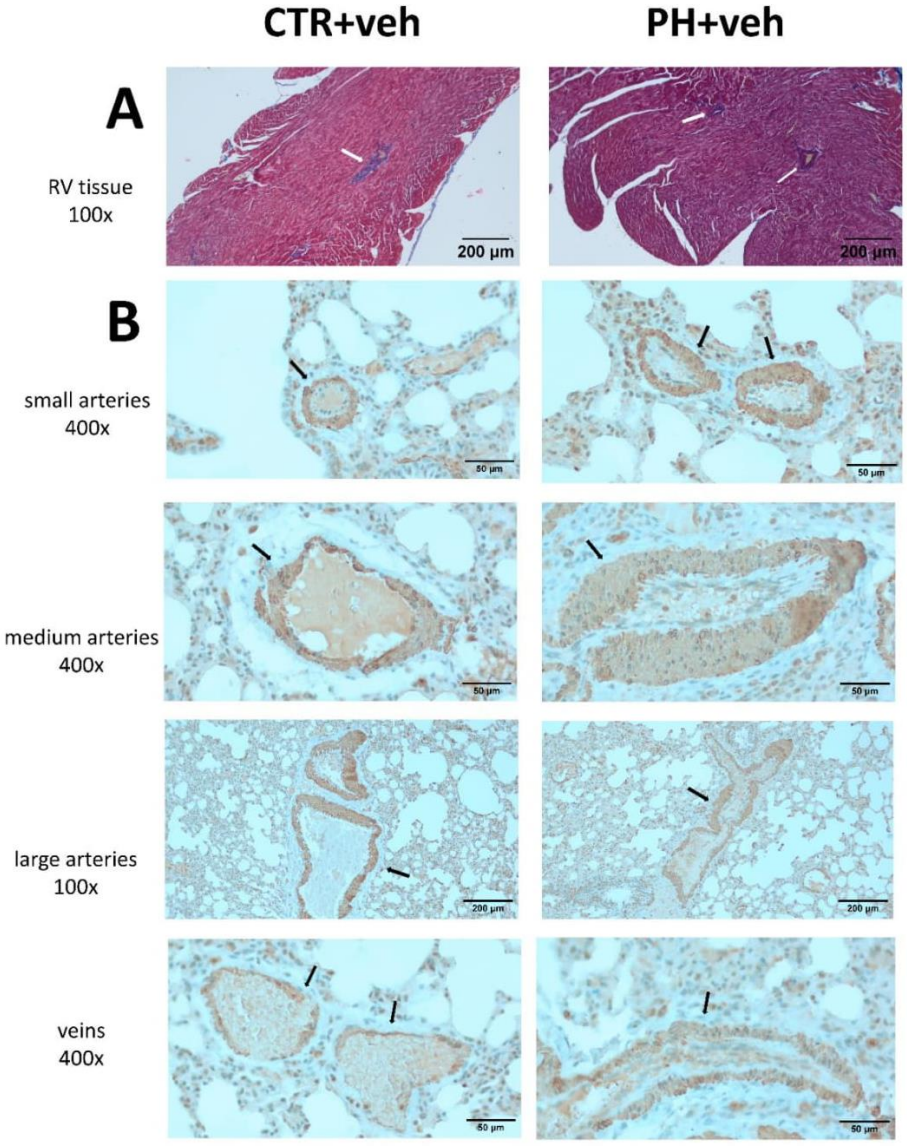
Supplementary Material



Supplementary Figure 1. Representative Masson trichrome-stained right ventricle (RV) images (**A**) and alpha smooth muscle actin immunohistochemistry-stained lung images of different types of vessels (**B**) of control (CTR+veh) and pulmonary hypertensive (PH+veh) animals (100x or 400x magnification, as indicated); quantitative evaluation is provided in Supplementary Table 1. Arrows indicate blood vessels.

Supplementary Table 1. Comparison of control (CTR+veh) and monocrotaline-induced pulmonary hypertensive (PH+veh) groups in lung and right ventricle (RV) histopathological and/or biochemical parameters. Only tendencies were observed.

Parameter	CTR+veh	*n*	PH+veh	*n*
Masson trichrome staining (%) in right ventricle				
- between bunches of cardiomyocytes	2.3 ± 0.3	7	2.5 ± 0.2	7
- around the coronary vessels	5.3 ± 1.0	7	8.8 ± 3.3	7
α smooth muscle actin staining scoring (0-4) in lungs				
- small arteries	2 (2;2)	7	2 (2;2)	7
- medium arteries	2 (2;3)	7	2 (2;3)	7
- large arteries	3 (2;3)	7	3 (3;3)	6
- veins	2 (2;3)	7	2 (2;2)	7
Collagen I Western blot (arbitrary units) in lungs	0.85 ± 0.09	5	1.0 ± 0.09	5

Monocrotaline (60 mg/kg) was injected s.c. at day 0. Tissues were isolated from animals on day 22. Data are presented as mean ± SEM of the percent area of the positive color reaction in Masson's trichromatic staining in the right ventricle, median with an interquartile range of scoring points in α smooth muscle actin staining in lungs and mean ± SEM of arbitrary units of collagen I/β-actin ratio in lungs. Scoring scale: absent expression (**0**); weak expression (**1**); moderate expression (**2**); high expression (**3**); very high expression (**4**).

1. Supplementary materials and methods

1.1 Histopathology

1.1.1 Alpha smooth muscle actin staining

Lungs were fixed with 10% buffered formalin. The tissue was paraffin-embedded and cross-sectioned at 5-µm thickness; sections were subjected to alpha smooth muscle actin (αSMA) immunohistochemical staining. Immunohistochemistry (IHC) grading based on intensity and frequency of staining results was performed by two independent investigators without knowledge of the clinicopathological features of the animals. The staining intensity was scored as negative (0), weak (+1), moderate (+2), or strong (+3). The frequency of positive cells in specific areas was scored as negative (0), less than 25% (+1), 25–50% (+2), 51–75% (+3), or more than 75% (+4). IHC grades were calculated by multiplying the intensity score by the frequency score as follows: −, absent expression (**0**); +, weak expression (**1**); ++, moderate expression (**2**); +++, high expression (**3**); or ++++, very high

expression (**4**). Vessels were assigned to the appropriate group according to their size. Large arteries – vessels of the lung cavity and their direct branches, visualized at x100 magnification or in the entire field of view at x400 magnification; medium arteries – vessels imaged at x400 magnification with an outer diameter of more than 1/3 of the field of view; lumen to wall thickness ratio greater than 1:1 in favor of lumen; small arteries – vessels imaged at x400 magnification with the morphology of thick-walled resistance vessels; outer diameter of the vessel less than 1/3 of the field of view; a lumen to wall thickness ratio of less than 1:1 in favor of the vessel wall; veins – vessels with a typical vein morphology, mainly imaged under x400 magnification.

1.1.2 Masson trichrome staining

As part of the histological technique, sections of right ventricle tissue were stained with Masson's trichromatic staining for collagen of connective tissue, using the kit Masson Trichrome with aniline blue (Bio-Optica, Milan, Italy; cat. no. 40211). The interstitial collagen volume fraction (CVF) in the myocardium (defined as the area of the positive color reaction in Masson's trichromatic staining (pink-purple) surface) was quantified in two localizations: 1) between bunches of cardiomyocytes and 2) around the coronary vessels to the total area of myocardium. The parameter is given as a percentage of positive color reaction staining in the entire surface of the myocardium. The described measurements were performed on histological photos of myocardium taken in a light microscope Olympus BX41 (Tokyo, Japan) with an Olympus DP12 camera magnification of 100 (x10 at the lens and x10 at the eyepiece). Morphometric measurements were made in the Zen 3.0 program (Carl Zeiss, Oberkochen, Germany) (Blue edition).

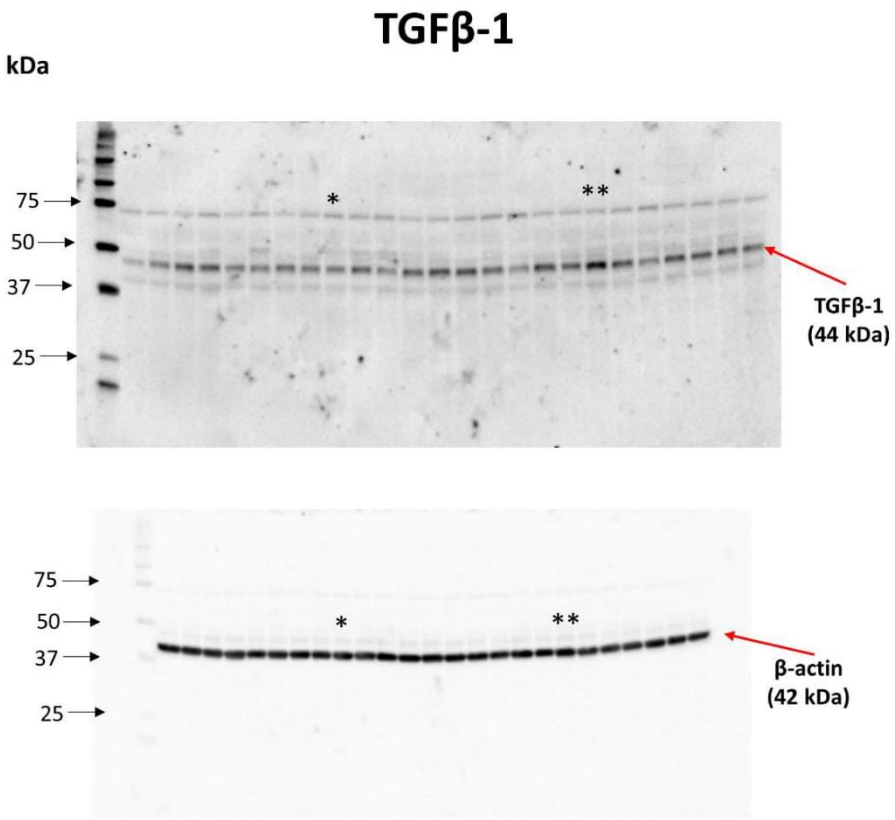
1.1 Western blot

The same procedure of the Western blot technique was used as in the main text of the article, except for not using a reducing agent in the sample buffer. The anti-collagen I antibody (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no. sc-293182) was used in a dilution of 1:750.

Western blot original images

Sample order:

- standard (row 1)
- CTR+veh (rows 2, 7, 12, 17, 22)
- PH+veh (rows 3, 8, 13, 18, 23)
- PH+MET (rows 4, 9, 14, 19, 24)
- PH+JD (rows 5, 10, 15, 20, 25)
- PH+JD+MET (rows 6, 11, 16, 21, 26)

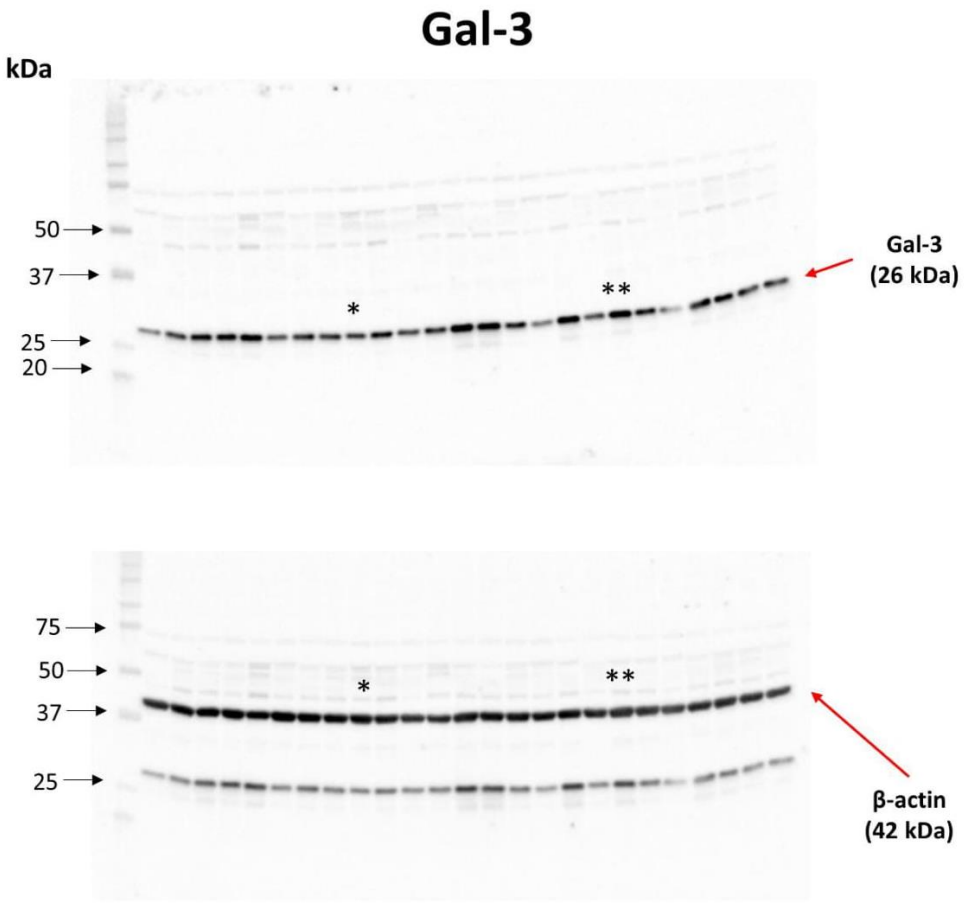


Supplementary Figure 2. Original images of Western blot analysis of transforming growth factor β-1 (TGFβ-1) and β-actin (loading control). The 10th and 20th rows are marked by * and **, respectively.

Western blot original images

Sample order:

- standard (row 1)
- CTR+veh (rows 2, 7, 12, 17, 22)
- PH+veh (rows 3, 8, 13, 18, 23)
- PH+MET (rows 4, 9, 14, 19, 24)
- PH+JD (rows 5, 10, 15, 20, 25)
- PH+JD+MET (rows 6, 11, 16, 21, 26)



Supplementary Figure 3. Original images of Western blot analysis of galectin-3 (Gal-3) and β-actin (loading control). The 10th and 20th rows are marked by * and **, respectively.

Chapter 12. Approvals of the Local Ethical Committee for Animal Experiments

UCHWAŁA NR 80/2017

z dnia 28.11.2017.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Olsztynie.

§ 1

Na podstawie art. 48 pkt. 1 ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266) po rozpatrzeniu wniosku pt.: **„Kompleksowa ocena wpływu kannabidiolu na układ krążenia, stres oksydacyjny i metabolizm serca w nadciśnieniu pierwotnym i wtórnym"** dnia 16.11.2017, złożonego przez **Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej, nr 0019 adres: ul. Mickiewicza 2 D, 15-089 Białystok**,[1] zaplanowanego przez **prof. dr hab. Barbarę Malinowską**[2] lokalna komisja etyczna:

WYRAŻA ZGODĘ[3]

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku **82/2017**

§ 2

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

1. Wniosek należy przypisać do kategorii: **Badania podstawowe, rodzaj:** kategoria sercowo-naczyniowy układ krążenia krwi i limfy
2. Najwyższy stopień dotkliwości proponowanych procedur to: **umiarkowana**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków[4]: 370 szt, **Szczur wędrowny (Rattus norvegicus),** stado **Cmdb:WI, 9-10 tygodni, 250-280 g i 6-7 tygodni, 180-210 g; 100 szt., Szczur wędrowny (Rattus norvegicus); stado SHR/NHsd, wiek 9-10 tygodni, masa ciała 220-250 g; 100 szt., Szczur wędrowny (Rattus norvegicus); stado WKY/NCrl, wiek 9-10 tygodni, masa ciała 250-280 g;**
4. Doświadczenia będą przeprowadzane przez: **Barbara Malinowska, Hanna Kozłowska, Marta Baranowska- Kuczko, Anna Pędzińska-Betiuk, Monika Kloza, Marek Toczek, Jolanta Weresa, Olga Karpińska, Rafał Kossakowski, Krzysztof Mińczuk, Irena Malinowska**
5. Doświadczenie będzie przeprowadzane w terminie[5] od **01.01.2018.** do **31.12.2020.**
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: **nie dotyczy**
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w: **Zakład Fizjologii i Patofizjologii Doświadczalnej, Wydz. Farm. UMB, ul. Mickiewicza 2A, Białystok, 15-089.**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ..... , w sposób: **nie dotyczy**
9. Doświadczenie **nie zostanie**[7] poddane ocenie retrospektywnej w terminie:

---

[1] 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;
[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Niewłaściwy zapis usunąć
[4] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik
[7] Niewłaściwy zapis usunąć

§ 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 **prof. dr hab. Barbara Malinowska**

§ 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

Podpisy przewodniczącego komisji

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

Otrzymuje Użytkownik

Pouczenie:

Od decyzji komisji można wnieść odwołanie do Krajowej Komisji Etycznej w terminie 14 od dnia otrzymania uchwały.

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UCHWAŁA NR 74/2020

z dnia 16.12.2020 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.: **„Ocena wpływu obwodowego antagonisty receptorów kannabinoidowych CB1 AM654 oraz aktywatora AMPK metforminy w doświadczalnym modelu tętniczego nadciśnienia płucnego u szczurów w mono i politerapii."** z dnia 02.12.2020 r., złożonego przez **Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej (0019)**, adres: **ul. Mickiewicza 2 D, 15-222 Białystok**, zaplanowanego przez **Barbara Malinowska**[2], przy udziale[3] (nie dotyczy) Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ[4]

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku **71/2020**.

§ 2

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

1. Wniosek należy przypisać do kategorii: **: Badania podstawowe. Sercowo-naczyniowy układ krążenia krwi i limfy (PB2).**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **dotkliwa**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków: **140 szt., szczur wędrowny (Rattus norvegicus), stado niekrewniacze Wistar Cmdb:Wi (samiec), 6-8 tyg.**
4. Doświadczenia będą przeprowadzane przez: **Malinowska Barbara, Hanna Kozłowska, Marta Baranowska- Kuczko, Anna Pędzińska-Betiuk, Jolanta Weresa, Monika Kloza, Bernadetta Gajo, Malinowska Irena, Aleksandra Kicman.**
5. Doświadczenie będzie przeprowadzane w terminie[5] **od 15.01.2021 do 31.12.2022**.
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: **nie dotyczy**
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w: **nie dotyczy**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ....., w sposób: **nie dotyczy**
9. Doświadczenie **zostanie** poddane ocenie retrospektywnej.

---

[1] Niewłaściwy zapis usunąć
[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[4] Niewłaściwy zapis usunąć
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik

§ 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 **Barbara Malinowska.**

§ 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. Krzysztof Wąsowicz

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.

UCHWAŁA NR 9/WNP/WDO/2021

z dnia 17.02.2021

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Olsztynie

§ 1

Lokalna komisja etyczna po rozpatrzeniu wniosku nr 10/2021 pt.: **„Ocena wpływu obwodowego antagonisty receptorów kannabinoidowych CB1 AM654 oraz aktywatora AMPK metforminy w doświadczalnym modelu tętniczego nadciśnienia płucnego u szczurów w mono i politerapii"** z dnia 09.02.2021 roku, złożonego przez **Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej (0019)**, adres **ul. A. Mickiewicza 2 D, 15-222 Białystok**, zaplanowanego przez **Barbara Malinowska**[1] a dotyczącego:

zmian w procedurach doświadczalnych

dodatkowych osób przeprowadzających doświadczenia

w ramach wydanej przez komisję zgody LKE w Olsztynie **uchwałą nr 74/2020 w dn. 16.12.2020 r**.

WYRAŻA ZGODĘ[2]

na dokonanie zmian w zakresie określonym poniżej.

§ 2

1. Najwyższy stopień dotkliwości proponowanych procedur na dodatkowych zwierzętach: **dotkliwa.**
2. Liczbę zwierząt wykorzystanych w doświadczeniu rozszerza się o: **nie dotyczy.**
3. Do Zespół prowadzący doświadczenia rozszerza się o następujące osoby (nazwisko i imię, nazwa użytkownika): **Patryk Remiszewski, Krzysztof Mińczuk Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej**
4. Doświadczenie będzie przeprowadzane w terminie[3]- **bez zmian**.

[1] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[2] Niewłaściwy zapis usunąć
[3] Nie dłużej niż 5 lat

§ 3

Uzasadnienie: Po dokonaniu oceny doświadczenia 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, zezwala na udział w eksperymencie dodatkowych osób oraz modyfikację procedury. Osobą odpowiedzialną za przeprowadzenie badań zgodnie z procedurami opisanymi we wniosku jest **Barbara Malinowska**.

§ 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
10-719 Olsztyn, ul. Oczapowskiego 13/4

Podpisy przewodniczącego komisji

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej
do Spraw Doświadczeń na Zwierzętach
prof. dr hab. Krzysztof Wąsowicz

Otrzymuje Użytkownik

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.

UCHWAŁA NR 39/WNP/2021

z dnia 21.04.2021

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Olsztynie

§ 1

Lokalna komisja etyczna po rozpatrzeniu wniosku nr 38/2021/WNP pt.: **„Ocena wpływu obwodowego antagonisty receptorów kannabinoidowych CB_1 AM654 oraz aktywatora AMPK metforminy w doświadczalnym modelu tętniczego nadciśnienia płucnego u szczurów w mono i politerapii"** z dnia 20.04.2021 roku, złożonego przez **Uniwersytet Medyczny w Białymstoku, Wydział Farmaceutyczny z Oddziałem Medycyny Laboratoryjnej (0019)**, adres **ul. Mickiewicza 2 D, 15-222 Białystok**, zaplanowanego przez **Barbara Malinowska**[1] a dotyczącego:

zmian w procedurach doświadczalnych

w ramach wydanej przez komisję zgody LKE w Olsztynie **uchwałą nr 74/2020 w dn. 16.12.2020 r.**

WYRAŻA ZGODĘ[2]

na dokonanie zmian w zakresie określonym poniżej.

§ 2

1. Najwyższy stopień dotkliwości proponowanych procedur na zwierzętach: **dotkliwa**
2. Liczbę zwierząt wykorzystanych w doświadczeniu rozszerza się o: **nie dotyczy**
3. Zespół prowadzący doświadczenia rozszerza się o następujące osoby (nazwisko i imię, nazwa użytkownika): **nie dotyczy**
4. świadczenie będzie przeprowadzane w terminie[3]- **bez zmian**.

[1] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[2] Niewłaściwy zapis usunąć
[3] Nie dłużej niż 5 lat

§ 3

Uzasadnienie: Po dokonaniu oceny doświadczenia 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, zezwala na dodanie procedury. Osobą odpowiedzialną za przeprowadzenie badań zgodnie z procedurami opisanymi we wniosku jest **Barbara Malinowska**.

§ 4

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

(Pieczęć lokalnej komisji etycznej)

Podpisy przewodniczącego komisji

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej
do Spraw Doświadczeń na Zwierzętach
prof. dr hab. Krzysztof Wąsowicz

.....

Otrzymuje Użytkownik

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.

Chapter 13. Author's statement

Białystok, 12 September 2022

Patryk Remiszewski
Name and surname of the author

Department of Experimental Physiology
and Pathophysiology
Medical University of Bialystok
Place of work/affiliation

Author's statement

I declare that my contribution in the preparation of the publications:

1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of my doctoral dissertation consisted of participation in chronic experiments (induction of hypertension and administration of cannabidiol to animals), measurement of physiological parameters, collecting and statistical analysis of data, visualization, writing, reviewing and editing of the original draft, which I define as 50% of the preparation of the above-mentioned publication.

2. *Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K., Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613, doi: 10.3389/fphar.2022.965613.*

which forms part of my doctoral dissertation consisted of the induction of pulmonary hypertension and the administration of tested compounds to animals, collecting and statistical analysis of data, visualization, writing, reviewing and editing of the original draft, which I define as 70% of the preparation of the above-mentioned publication.

3. *Remiszewski P.; Malinowska B. Why multitarget vasodilatory (endo)cannabinoids are not effective as antihypertensive compounds after chronic administration: Comparison of their effects on systemic and pulmonary hypertension. Pharmaceuticals 2022, 15, 1119, doi: 10.3390/ph15091119.*

which forms part of my doctoral dissertation consisted of conceptualization, writing, reviewing and editing of the original draft, which I define as 75% of the preparation of the above-mentioned publication.

Patryk Remiszewski
.....
Author's signature

.....
Supervisor's signature

Chapter 14. Co-authors' statements

Białystok, 12 September 2022

Prof. Dr. Barbara Malinowska
Name and surname of the co-author

Department of Experimental Physiology
and Pathophysiology
Medical University of Bialystok
Place of work/affiliation

Co-author's statement

I declare that my contribution in the preparation of the publications:

1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of conceptualization, writing, review and editing of the original draft, supervision and project administration.

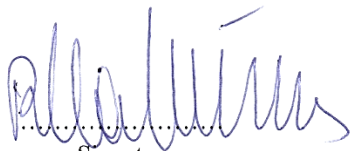
2. *Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K., Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613, doi: 10.3389/fphar.2022.965613.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of conceptualization, review of the original draft and supervision of the project.

3. *Remiszewski P.; Malinowska B. Why multitarget vasodilatory (endo)cannabinoids are not effective as antihypertensive compounds after chronic administration: Comparison of their effects on systemic and pulmonary hypertension. Pharmaceuticals 2022, 15, 1119, doi: 10.3390/ph15091119.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of conceptualization, writing and editing of the original draft.

At the same time, I agree to submit the above-mentioned papers by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.



.....
Signature

Rheinische Friedrich-Wilhelms-Universität

Institut für Pharmakologie und Toxikologie
Direktor: Prof. Dr. Alexander Pfeifer

Universitätsklinikum Bonn

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e.schlicker@uni-bonn.de

Bonn, August 13, 2022

Co-author's statement

I declare that my contribution in the preparation of the publications:

1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*
2. *Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K., Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613 doi: 10.3389/fphar.2022.965613.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of reviewing and editing the original drafts.

At the same time, I agree that the above-mentioned papers by Mr. Patryk Remiszewski will be submitted as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

Eberhard Schlicker

Prof. Dr. Eberhard Schlicker

Białystok, 2 August 2022

Dr. Anna Pędzińska-Betiuk
Name and surname of the co-author

Department of Experimental Physiology
and Pathophysiology
Medical University of Bialystok
Place of work/affiliation

Co-author's statement

I declare that my contribution in the preparation of the publications:

1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing formal analysis and visualization.

2. *Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K., Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613 doi: 10.3389/fphar.2022.965613.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing experiments and reviewing the original draft.

At the same time, I agree to submit the above-mentioned papers by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

Anna Pędzińska-Betiuk
Signature

Białystok, 30 August 2022

Dr. Iwona Jarocka-Karpowicz
Name and surname of the co-author

Department of Analytical Chemistry
Medical University of Białystok
Place of work/affiliation

Co-author's statement

I declare that my contribution in the preparation of the publication:

1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing biochemical analyses.

At the same time, I agree to submit the above-mentioned paper by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

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1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

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1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

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1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing the experiments and visualization.

At the same time, I agree to submit the above-mentioned paper by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

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1. *Remiszewski, P.; Jarocka-Karpowicz, I.; Biernacki, M.; Jastrząb, A.; Schlicker, E.; Toczek, M.; Harasim-Symbor, E.; Pędzińska-Betiuk, A.; Malinowska, B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing Western blot analyses.

At the same time, I agree to submit the above-mentioned paper by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

Harasim-Symbor Ewa
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1. *Remiszewski P.; Pędzińska-Betiuk A.; Mińczuk K., Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613 doi: 10.3389/fphar.2022.965613.*

which forms part of the doctoral dissertation of Mr. Patryk Remiszewski consisted of performing experiments.

At the same time, I agree to submit the above-mentioned paper by Mr. Patryk Remiszewski as a part of his doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.

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.....
Signature

Chapter 15. Scientific achievements

Total Impact Factor (IF): 19.380

Total Ministry of Education and Science (MES) points: 365

List of publications constituting the doctoral dissertation

1. **Remiszewski P.**; Jarocka-Karpowicz I.; Biernacki M.; Jastrząb A.; Schlicker E.; Toczek M.; Harasim-Symbor E.; Pędzińska-Betiuk A.; Malinowska B. Chronic cannabidiol administration fails to diminish blood pressure in rats with primary and secondary hypertension despite its effects on cardiac and plasma endocannabinoid system, oxidative stress and lipid metabolism. Int J Mol Sci 2020, 21, 1295, doi:10.3390/ijms21041295. (IF) = 5.924, (MES) = 140 pts.

2. **Remiszewski P.**; Pędzińska-Betiuk A.; Mińczuk K.; Schlicker E.; Klimek J.; Dzięcioł J.; Malinowska B. Effects of the peripheral CB_1 receptor antagonist JD5037 in mono- and polytherapy with the AMPK activator metformin in a monocrotaline-induced rat model of pulmonary hypertension. Front Pharmacol 2022, 13, 965613, doi: 10.3389/fphar.2022.965613. (IF) = 5.988, (MES) = 100 pts.

3. **Remiszewski P.**; Malinowska B. Why multitarget vasodilatory (endo)cannabinoids are not effective as antihypertensive compounds after chronic administration: Comparison of their effects on systemic and pulmonary hypertension. Pharmaceuticals 2022, 15, 1119, doi: 10.3390/ph15091119. (IF) = 5.215, (MES) = 100 pts.

List of other scientific publications

1. Biernacki M.; Malinowska B.; Timoszuk M.; Toczek M.; Jastrząb A.; **Remiszewski P.**; Skrzydlewska E. Hypertension and chronic inhibition of endocannabinoid degradation modify the endocannabinoid system and redox balance in rat heart and plasma. Prostaglandins Other Lipid Mediat 2018, 138, 54-63, doi: 10.1016/j.prostaglandins.2018.09.001. (IF) = 2.253, (MES) = 25 pts.

List of conference reports

1. **Remiszewski P.**, Pędzińska-Betiuk A., Mińczuk K., Weresa J., Krzyżewska A., Malinowska B. Combined AMPK activation and CB_1 receptor blockade as a new target in pulmonary arterial hypertension treatment. 3rd Baltic Pulmonary Hypertension and Circulation Conference. Tallinn, Estonia (Online), 01.10.2021.

2. **Remiszewski P.**, Pędzińska-Betiuk A., Mińczuk K., Weresa J., Krzyżewska A., Malinowska B. Effects of peripheral cannabinoid CB_1 receptor inverse agonist JD5037 in mono- and polytherapy with metformin in a monocrotaline-induced rat model of pulmonary arterial hypertension. 28th Congress of the Polish Physiological Society. Gdansk, Poland (Online). September 15-17, 2021.

3. **Remiszewski P.**, Toczek M., Biernacki M., Timoszuk M., Jastrząb A., Malinowska B. Kannabidiol modyfikuje parametry stresu oksydacyjnego w sercu i osoczu szczurów z nadciśnieniem pierwotnym i wtórnym. XXIV Sympozjum Sekcji Kardiologii Eksperymentalnej Polskiego Towarzystwa Kardiologicznego. Tomaszowice k. Krakowa, 28-30.11.2019.

4. Malinowska B., Pędzińska-Betiuk A., Toczek M., Biernacki M., Timoszuk M., Jastrząb A., Weresa J., **Remiszewski P.** Infuence of chronic cannabidiol administration on cardiovascular parameters, endocannabinoid levels and oxidative stress in spontaneously hypertensive and normotensive rats. 29th Annual Symposium of the International Cannabinoid Research Society, Bethesda, Maryland, USA, June 29 - July 4, 2019.

5. **Remiszewski P.**, Toczek M. Physiological effects of chronic inhibition of endocannabinoids degradation in spontaneously hypertensive rats. 11th BIMC Bialystok International Medical Congress for Young Scientists, Bialystok, Poland, May 5-7th, 2016.

6. **Remiszewski P.**, Semeniuk A., Toczek M. Wpływ przewlekłego podania inhibitora rozkładu endokannabinoidów w doświadczalnym modelu pierwotnego nadciśnienia tętniczego u szczurów. IX Konferencja Adeptów Fizjologii, Gdańsk, 15-16 października 2015 r.

7. Semeniuk A., **Remiszewski P.**, Toczek M. Zależne od wieku efekty hamowania rozkładu endokannabinoidów w doświadczalnym modelu wtórnego nadciśnienia tętniczego u szczura. IX Konferencja Adeptów Fizjologii, Gdańsk, 15-16 października 2015 r.

8. **Remiszewski P.**, Janowski H., Semeniuk A. Impairment of neurogenic vasopressor response in DOCA-salt hypertensive rats. 10th BIMC Bialystok International Medical Congress for Young Scienctists, Bialystok, May 14-16th 2015.

9. **Remiszewski P.**, Semeniuk A. Zastosowanie kannabinoidów we współczesnej farmakoterapii. Ogólnopolska Konferencja Naukowa Farmakoterapia Kobiet w Ciąży i Elementy Farmakoekonomiki Białystok, 25.04.2015 r.

10. Semeniuk A., **Remiszewski P.** Testy diagnostyczne dostępne w aptece. Ogólnopolska Konferencja Naukowa Farmakoterapia Kobiet w Ciąży i Elementy Farmakoekonomiki Białystok, 25.04.2015 r.

11. Semeniuk A., **Remiszewski P.**, Janowski H. Wpływ zahamowania rozkładu endogennych kannabinoidów na wybrane parametry fizjologiczne u szczura z nadciśnieniem DOCA-salt. I Ogólnopolska Konferencja Studentów Medycyny Laboratoryjnej "Młodzi Diagności w Łodzi", Łódź, 15-16 listopada 2014.

12. **Remiszewski P.**, Semeniuk A., Janowski H. Effects of the chronic administration of the fatty acid amide hydrolase inhibitor URB597 on some physiological parameters in the desoxycorticosterone acetate (DOCA)-salt hypertensive rats. 9th Bialystok International Medical Congress for Young Scientists. Bialystok, 24-26th April 2014.

13. Semeniuk A., Janowski H., **Remiszewski P.** Advantages and disadvantages of the desoxycorticosterone acetate (DOCA)-salt hypertension model in Wistar rats. 9th Bialystok International Medical Congress for Young Scientists. Bialystok, 24-26th April 2014.

14. Janowski H., **Remiszewski P.**, Semeniuk A. Effects of chronic the fatty acid amide hydrolase inhibitor - URB 597 on blood pressure, heart and kidney weight

in DOCA-salt hypertensive rats. 9th Bialystok International Medical Congress for Young Scientists. Bialystok, 24-26th April 2014.