Letter to the Editor

Metformin inhibits both oleic acid-induced and CB1R receptor agonist-induced lipid accumulation in Hep3B cells: The preliminary report

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Abstract
Fatty liver is characterized by excessive accumulation of triglycerides within hepatocytes. Recent findings indicate that natural history of nonalcoholic fatty liver is regulated, in part, by endogenous cannabinoids. Metformin is an oral hypoglycemic medication which inhibits gluconeogenesis and glycogenolysis in hepatocytes and limits lipid storage in the liver through the inhibition of free fatty acid formation via induction of activated protein kinase activity (AMPK). Both endocannabinoids and metformin may modulate hepatosteatosis; therefore, it was interesting to examine whether metformin may affect lipid accumulation in hepatocytes by acting on cannabinoid receptors, CB1 and CB2, in vitro study. Hep3B cells were incubated with or without metformin (Met), phosphatidylcholine (PC), and oleic acid (OA). Cells without any of the examined substances served as negative control. Cells treated only with OA served as positive control. The quantity of intracellular lipids was assessed using Oil-Red-O staining. Selective CB1R agonist, arachidonyl-2-chloromethylamide (ACEA), and CB2R agonist, AM1241 (2-iodo-5-nitrophenyl)-[1-(methylpiperidin-2-ylmethyl)-1H-indol-3-yl]methanone, were also used to treat Hep3B cells. In some experiments, antagonist for CB1R, AM6545, or SR144528 as selective antagonist of CB2R were used. In the study, Met decreased lipid accumulation in cells treated with OA and inhibited CB1R agonist–induced lipid accumulation in hepatocytes. The CB2R agonist–induced hepatic lipid accumulation was not inhibited by metformin. The results indicate that metformin may interact with endocannabinoid system in the liver by inhibiting CB1R agonist–stimulated fat accumulation in hepatocytes.

Keywords
cannabinoids, fatty liver, metformin

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Introduction
Fatty liver is characterized by excessive accumulation of triglycerides within hepatocytes. It is an initial stage of many liver diseases including nonalcoholic fatty liver disease (NAFLD). Mechanisms involved in hepatosteatosis include activation of lipogenic transcription factor sterol regulatory element-binding protein 1c (SREBP1c), impaired function of lipolytic transcription factor peroxisome proliferator-activated receptor alpha (PPARalpha), and inhibited AMP-activated protein kinase

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(AMPK) activity as well.\textsuperscript{1–3} Recent findings indicate that NAFLD is regulated partially by endogenous cannabinoid (EC).\textsuperscript{4} Cannabinoid receptors are localized mainly in the brain, but cannabinoid receptor 1 (CB1R) is also present in the liver and in other peripheral tissues and cannabinoid receptor 2 (CB2R) in immune and hematopoietic cells. The liver expresses CB1R and produces endocannabinoids which regulate hepatic lipid metabolism and are involved in the development of NAFLD.\textsuperscript{4} It was reported that overactivation of peripheral CB1R plays a vital role in liver steatosis in experimental animal model of NAFLD.\textsuperscript{5}

Recent evidence suggests, however, that the cannabinoids $\Delta^9$-tetrahydrocannabivarin (THCV) and cannabidiol (CBD) improve insulin sensitivity and directly reduce accumulated lipid levels in vitro in a hepatosteatosis model and adipocytes. Nuclear magnetic resonance (NMR)–based metabolomics confirmed these results and further identified specific metabolic changes in THCV and CBD-treated hepatocytes. Treatment also induced post-translational changes in a variety of proteins such as CREB, PRAS40, AMPKa2, and several STATs indicating increased lipid metabolism and, possibly, mitochondrial activity. However, it is not known if these cannabinoids act as agonists or antagonists of cannabinoid receptors.\textsuperscript{6}

Metformin is an oral hypoglycemic medication which not only inhibits gluconeogenesis and glycogenolysis in hepatocytes but also uses various mechanisms to restore insulin sensitivity, for example, by limiting lipid storage in the liver through the inhibition of free fatty acid formation via induction of AMPK. AMPK suppresses acetyl-CoA carboxylase and HMG-CoA reductase and therefore decreases fatty acid synthetase expression. In addition, AMPK suppresses fatty acid synthesis through inhibition of SREBP-1c.\textsuperscript{7}

At the molecular level, the findings vary depending on the doses of metformin used and duration of treatment, with clear differences between acute and chronic administration. Metformin has been shown to act via both AMPK-dependent and AMPK-independent mechanisms; by inhibition of mitochondrial respiration but also perhaps by inhibition of mitochondrial glycerophosphate dehydrogenase, and a mechanism involving the lysosome. Both endocannabinoids and metformin may modulate hepatosteatosis; therefore, it was interesting to examine whether metformin may affect lipid accumulation in hepatocytes by acting on CB1R and CB2R in an in vitro study. We chose to use hepatoma cell line Hep3B, a classical tool in cell biology, which in vitro maintains the primary features of human hepatocytes.\textsuperscript{7}

**Material and methods**

Hep3B cells at the density of $1 \times 10^5$/mL in Eagle’s minimum essential medium (MEM) supplemented with 10% of fetal bovine serum were seeded in 24-well plastic plates (Nuncl), 1mL per well. One day later, cells were incubated (and) without or with metformin (Sigma). Two concentrations of metformin were used: 20 or 100$\mu$M. Similarly, another group of cells was incubated (and) without or with phosphatidylcholine (PC) (Sigma) in two concentrations: 20 or 100$\mu$g/mL. Into some wells oleic acid (OA) (Sigma) at final 50$\mu$M concentration was added. Stock solution of OA and PC was prepared in dimethyl sulfoxide and metformin in cell culture medium. Cells without any of substances examined served as negative control. Cells treated only with OA served as positive control. In some experiments, vehicle used to prepare stock solution at different concentrations was also examined. Cells were incubated at 37°C (5% CO$_2$) for 7 days. The medium with substances examined was changed every 2 days. After removing of medium and washing with phosphate buffered saline into wells, 0.5 mL of 10% formalin was added and incubated 30 min at room temperature. Then, staining of intracellular neutral lipids was performed using Oil-Red-O (Sigma) according to the manufacturer instructions. Optical density (OD) of samples was measured at 490 nm in VICTOR X4 Multilabel Plate Reader (Perkin Elmer).

Selective CB agonists were also used to treat Hep3B cells over 7 days. Arachidonyl-2-chloromethylamide (ACEA) (Sigma), a high affinity CB1R agonist was added to cell cultures in concentration of 12nM. AM1241 (2-iodo-5-nitrophenyl)-[1-(methylpiperidin-2-ylmethyl)-1H-indol-3-yl]methaneone (Sigma) was used as a selective CB2R agonist at concentration of 16$\mu$M. In some experiments, antagonist for CB1R, AM6545 (5-(4-[4-cyanobut-1-ynyl]phenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(1,1-dioxo-thiomorpholino)-1H-pyrazole-3-carboxamide) (Sigma) in concentration of 1$\mu$M, or SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan-2-y]-5-(4-chloro-3
-methylphenyl)-1-[(4-methylphenyl)methyl]-1\textit{H}-pyrazole-3-carboxamide) (Cayman Chemicals) as selective antagonist of CB2R at concentration of 1 µM was used. Stock solution of agonists and antagonists was prepared in alcohol. Three independent experiments were conducted with eight replicates of each. Results are presented as a mean ± SD. Comparison between groups was performed by one-way analysis of variance (ANOVA) and post hoc Tukey’s with Bonferroni correction (Statistica, StatSoft Inc., Tulsa, USA).

### Results and discussion

As can be seen from Table 1, OA-induced lipid accumulation in cells, while metformin and PC independently of their concentrations, inhibited the process. However, the results were statistically significant, only for higher concentrations of metformin and phosphatidylcholine. These results confirm the results achieved by other authors, who detected that both mentioned substances suppress lipid accumulation in hepatocytes in vitro and in liver of experimental animals as well as in humans.\(^8,9\) These results also confirm that Hep3B cells could be used as model for examination of influence of different substances on lipid accumulation in cells.

In order to detect which cannabinoid receptors are involved in lipid accumulation in the presence of OA as inducer, the cells were treated with OA in the presence of agonists and antagonists of cannabinoid receptors. It can be seen from Table 2 that antagonist of CB2R inhibited significantly accumulation of lipids in Hep3B cells. The result obtained strongly indicates that OA-induced lipid accumulation in hepatocytes cooperates with CB2R. In contrast to that, agonist of CB1R significantly enhanced lipid accumulation in cells when used alone or together with OA. These results also indicate that both receptors are expressed in Hep3B cells. Moreover, we showed that Hep3B cells can reproduce the steatosis seen in animals and can be used to delineate its mechanism. Therefore, they could be used to examine which CB1R or CB2R are involved in the inhibition of lipid accumulation by metformin and PC (Table 3). These results confirm earlier paper in which it was detected that both receptors participated in the regulation of lipid metabolism by regulating the expression of key enzymes of lipid synthesis and transport such as SREBP-1c and carnitine palmitoyltransferase 1.\(^7\)

The results obtained are in agreement with earlier results of De Gottardi et al.\(^{10\text{a}}\) The authors detected that in OA-treated HepG2 cells, both CB1R and CB2R agonists increased in dose-dependent manner degree of steatosis. Therefore, they stated that both CB1R and CB2R participate in the regulation of lipid metabolism by regulating the expression of key enzymes of lipid synthesis and transport such as carnitine palmitoyltransferase 1 and SREBP-1c. Recent evidence suggests, however, that the cannabinoids THCV and CBD directly reduce accumulated lipid levels in vitro in a hepatosteatosis model and adipocytes. Treatment also induced post-translational changes in a variety of proteins such as CREB, PRAS40, AMPKa2, and several STATs indicating increased lipid metabolism and, possibly, mitochondrial activity. However, it is not known, if these cannabinoids act as agonists or antagonists cannabinoid receptors.\(^6\)

In order to examine whether metformin and PC can inhibit lipid accumulation in liver cells, Hep3B cells were treated with OA in the presence of metformin or PC. Moreover, cells were treated with agonists of CB1R and CB2R for 7 days. Analysis of fat accumulation in the cells revealed that metformin decreased lipid accumulation in cells treated only with OA. Moreover, CB1R agonist significantly increased fat accumulation while

### Table 1. Effects of metformin and PC on OA-induced lipid accumulation in Hep3B cells.

| Substances | % OD of negative control | % OD of positive control |
|------------|--------------------------|--------------------------|
| Metformin 20 µM | 90.8 ± 2.32 | 82.6 ± 2.1 |
| Metformin 100 µM | 91.0 ± 3.3 | 73.4 ± 1.9\(^a\) |
| PC 20 µg/mL | 102.9 ± 4.6 | 88.5 ± 1.62 |
| PC 100 µg/mL | 97.7 ± 4.5 | 78.1 ± 2.31\(^a\) |
| OA | 135.3 ± 2.96\(^b\) | – |

PC: phosphatidylcholine; OA: oleic acid; OD: optical density.
\(^a\)Statistically significant compared with positive control (OA), \(P \leq 0.05\).
\(^b\)Statistically significant compared with negative control, \(P \leq 0.05\).
Table 2. Oleic acid as enhancer of lipid accumulation in Hep3B cells induced by agonists of cannabinoid receptors.

**CB1R**

| Substances | % OD of negative control | % OD of sample with OA | % OD of sample with ACEA |
|------------|--------------------------|------------------------|--------------------------|
| OA         | 132.2 ± 2.6^a            | –                      | –                        |
| Vehicle    | 1.6 ± 0.2                | –                      | –                        |
| OA + ACEA  | 165.7 ± 3.3^a            | 121.0 ± 1.8^b          | 148.9 ± 5.0              |
| OA + AM6545| 149.6 ± 2.9^a            | 99.6 ± 2.45            | –                        |
| ACEA       | 134.5 ± 1.88^a           | –                      | –                        |
| AM6545     | 96.8 ± 3.7               | –                      | –                        |
| Vehicle    | 1.2 ± 0.1                | –                      | –                        |

**CB2R**

| Substances | % OD of negative control | % OD of sample with OA | % OD of sample with AM1241 |
|------------|--------------------------|------------------------|----------------------------|
| OA         | 132.2 ± 2.6^a            | –                      | –                          |
| OA + AM1241| 136.15 ± 2.2^a           | 114.0 ± 3.1            | 103.4 ± 3.7               |
| OA + SR144528| 100.32 ± 3.0^c         | 69.1 ± 3.1^b           | –                          |
| AM1241     | 140.0 ± 3.8^a            | –                      | –                          |
| SR144528   | 101.0 ± 2.0              | –                      | –                          |
| Vehicle    | 0.2 ± 0.1                | –                      | –                          |

CB1R: cannabinoid receptor 1; CB2R: cannabinoid receptor 2; OD: optical density; OA: oleic acid; ACEA: arachidonyl-2-chloromethylamide—selective CB1R agonist; AM6545: 5-(4-cyanobut-1-ynyl)[phenyl]-1-(2,4-dichlorophenyl)-4-methyl-N-(1,1-dioxo-thiomorpholino)-1H-pyrazole-3-carboxamide—agonist CB1R; AM1241: (2-iodo-5-nitrophenyl)-[1-(methylpiperidin-2-ylmethyl)-1H-indol-3-yl]methanone—CB2R agonist; SR144528, N-[(15)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide—agonist CB2R.

^aStatistically significant compared with negative control, \( P \leq 0.05 \).

^bStatistically significant compared with positive control (OA), \( P \leq 0.05 \).

^cStatistically significant compared with OA and AM1241, \( P \leq 0.05 \).

Table 3. The influence of metformin and PC on lipid accumulation in Hep3B cells induced by oleic acid (positive control) or by agonists of cannabinoid receptors.

| Substances | % OD negative control | % OD sample with OA | % OD sample with agonists |
|------------|-----------------------|---------------------|--------------------------|
| OA         | 135.3 ± 3.6^a         | –                   | –                        |
| Met        | 93.1 ± 2.0             | –                   | –                        |
| Vehicle    | 0.2 ± 0.1              | –                   | –                        |
| OA + Met   | 111.0 ± 2.9            | 74.6 ± 1.7^b        | –                        |
| PC         | 98.7 ± 3.3             | –                   | –                        |
| Vehicle    | 0.1 ± 0.2              | –                   | –                        |
| OA + PC    | 113.1 ± 2.31           | 72.9 ± 3.2^b        | –                        |
| Met + ACEA | 115.6 ± 2.8            | –                   | 84.0 ± 2.5^b             |
| PC + ACEA  | 140.3 ± 2.8^a          | –                   | 105.6 ± 3.7              |
| ACEA       | 134.5 ± 1.88^a         | –                   | –                        |
| AM1241     | 140.0 ± 3.8^a          | –                   | –                        |
| Met + AM1241| 133.0 ± 5.2^a          | –                   | 95.2 ± 3.4               |
| PC + AM1241| 139.6 ± 4.4^a          | –                   | 99.8 ± 4.3               |

OA: oleic acid; OD: optical density; Met: metformin; PC: phosphatidylcholine; ACEA: arachidonyl-2-chloromethylamide—selective CB1R agonist; AM1241: (2-iodo-5-nitrophenyl)-[1-(methylpiperidin-2-ylmethyl)-1H-indol-3-yl]methanone—CB2R agonist. Selective CB agonists were used to treat Hep3B cells over 7 days. Arachidonyl-2-chloromethylamide (ACEA) (Sigma), a high affinity CB1R agonist, was added to cell cultures at concentration of 12 nM. AM1241 (2-iodo-5-nitrophenyl)-[1-(methylpiperidin-2-ylmethyl)-1H-indol-3-yl]methanone (Sigma) was used as a selective CB2R agonist at concentration of 16 nM. Vehicles used as controls were at concentration present in substance examined.

^aStatistically significant compared with negative control, \( P \leq 0.05 \).

^bStatistically significant compared with positive control (OA or ACEA), \( P \leq 0.05 \).
metformin inhibited this process. In contrast to that, CB2R agonist increased significantly lipid accumulation; however, metformin did not influence this process. In contrast to metformin, PC did not influence lipid accumulation induced by CB1R and CB2R agonists, indicating that its activity in decreasing lipid accumulation in hepatocytes is not connected with cannabinoid receptors.

Endocannabinoids, acting via CB1R, stimulate appetite in the hypothalamus but direct peripheral effects have also been observed in animal studies, thus implicating the role of endocannabinoids in lipogenesis. CB1R antagonist, rimonabant, inhibits food intake and in phase III clinical trials have shown effect on glucose and fat metabolism. Moreover, it has been found that cannabinoids stimulate AMPK activity in hypothalamus but inhibit AMPK action in the liver and adipose tissue. AMPK serves as an intracellular sensor for energy homeostasis and the AMPK pathway regulates also lipid and glucose metabolism. In hepatic and adipose tissue, CB1R agonist treatment has been demonstrated to inhibit AMPK activity. In contrast to that, metformin improves insulin resistance through activation of AMPK but also limits lipid storage through inhibition of free fatty acid formation and suppression of acetyl-CoA carboxylase and HMG-CoA reductase. There is no information in the literature concerning interactions between endocannabinoids and metformin; therefore, our discovery that metformin may inhibit lipid accumulation in hepatocytes by cooperation with CB1R needs further experiments. However, we expect that AMPK pathway regulation by endocannabinoids and metformin may be the main pathway involved in lipid storage in hepatocytes. It should be noted, however, that metformin itself is not a ligand for CB1R but influence its action. CB1R and CB2R are G-protein-coupled receptors (GPCRs), similar to receptors for many hormones and neurotransmitters. Recently, it was detected that metformin may modulate GPCR signaling system, especially in tumor cells. Moreover, rimonabant, a potent CB1 cannabinoid receptor antagonist, is a G protein inhibitor. Therefore, it is also possible that metformin may act on CB1R signaling system decreasing lipid accumulation in hepatocytes. Confirmation of this hypothesis needs further experiments.

In conclusion, OA-induced lipid accumulation in cells by affecting both cannabinoid receptors, to a greater extent CB1R. Metformin and PC used in both lower and higher concentration inhibited lipid accumulation induced by OA. PC reduced OA-induced lipid accumulation in cells, but not by interaction with cannabinoid receptors. Metformin reduced OA-induced lipid accumulation, not via CB2R, but due to interference with activity of CB1R. It seems likely that metformin along with several other discovered mechanisms may also interact with endocannabinoid system in the liver.

**Declaration of conflicting interests**
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**Ethical approval**
All the procedures described were carried out with approval and performed in accordance with the guidelines of the ethics committee of Medical University of Lublin, Poland

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