Additive effects of dexamethasone and palmitate on hepatic lipid accumulation and secretion

Ewa Harasim-Symbor, Karolina Konstantynowicz-Nowicka and Adrian Chabowski
Department of Physiology, Medical University of Białystok, Białystok, Podlaskie, Poland

Abstract
Synthetic and natural glucocorticoids are able to highly modify liver lipid metabolism, which is possibly associated with nonalcoholic fatty liver disease development. We have assessed the changes in lipid and sphingolipid contents in hepatocytes, lipid composition and saturation status as well as the expression of proteins involved in fatty acid transport after both dexamethasone and palmitate treatments. The experiments were conducted on primary rat hepatocytes, incubated with dexamethasone and/or palmitic acid during short (16 h) and prolonged (40 h) exposure. Intracellular and extracellular lipid and sphingolipid contents were assessed by gas liquid chromatography and high-performance liquid chromatography, respectively. The expression of selected proteins was estimated by Western blotting. Short and prolonged exposure to dexamethasone combined with palmitic acid resulted in increased expression of fatty acid transporters, which was subsequently reflected by excessive intracellular accumulation of triacylglycerols and ceramide. The expression of microsomal transfer protein and cassette transporter was also significantly increased after dexamethasone and palmitate treatment, which was in accordance with elevated extracellular lipid and sphingolipid contents. Our data showed additive effects of dexamethasone and palmitate on protein-dependent fatty acid uptake in primary hepatocytes, resulting in the increased accumulation of triacylglycerols and sphingolipids. Moreover, the combined treatment altered fatty acid composition and diminished triacylglycerols desaturation index. Importantly, we observed that additive effects on both increased microsomal transport protein expression as well as elevated export of triacylglycerols, which may be relevant as a liver protective mechanism.

Introduction
Dexamethasone (DEX) is one of the most commonly prescribed synthetic glucocorticoids with wide anti-inflammatory and immunosuppressive properties (Overman et al. 2013, Kumar et al. 2015). However, prolonged DEX treatment causes adverse side effects, including disturbances in glucose and lipid metabolism in different tissues, such as liver (Fardet et al. 2007). Glucocorticoids modulate hepatic fatty acid synthase and acetyl-CoA carboxylase genes expression resulting in de novo lipogenesis stimulation and increased fatty acid β-oxidation (Sørensen et al. 1992, Dolinsky et al. 2004, Wang et al. 2012a). Moreover, the above-mentioned metabolic disturbances together with the decreased VLDL secretion result in excessive accumulation of fatty
acids in hepatocytes (Woods et al. 2015). Chronic stress, evidently connected with modern lifestyle, modifies liver lipid metabolism and leads to increased concentration of cortisol, which subsequently activates adipose tissue lipolysis and increases the content of fatty acids in plasma (Han et al. 2015). In conclusion, the above-mentioned disturbances may be evoked by both endogenous and exogenous glucocorticoids, which play a pivotal role in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) (Morgan et al. 2014, Woods et al. 2015).

NAFLD represents the most common liver disease in Western modern societies (Woods et al. 2015). It is defined as increased accumulation of triacylglycerols (TAG) in cytoplasm of more than 5% of hepatocytes, which is typically a reversible situation (Szczepeński et al. 2005, Cohen et al. 2011). However, prolonged occurrence of steatosis may progress to inflammation and the indelible hepatic fibrosis namely nonalcoholic steatohepatitis (NASH). Furthermore, NASH being a progressive form of disease may deteriorate into cirrhosis and hepatocellular carcinoma (HCC) (Adams et al. 2005, Gan et al. 2015). However, it is still unclear whether the occurrence and development of steatosis is a cause or a consequence of hepatic metabolic dysfunctions. It is widely accepted that the incidence of NAFLD is strongly associated with obesity, and the most common feature of obese individuals is chronically elevated level of circulating fatty acids (Cusi 2012). Based on that, it seems plausible that the increased availability of fatty acids might be the primary cause of NAFLD. Modern diet, rich in fatty acids lacking carbon-to-carbon double bond, called saturated fatty acids, influences hepatocytes lipid metabolism and may lead to liver diseases (Kien et al. 2014). Moreover, both the amount of dietary lipids and its saturation status in plasma are important (McKinnime et al. 2013).

As mentioned previously, the changes in hepatocyte lipid metabolism are highly dependent on plasma long-chain fatty acids (LCFAs) availability, and LCFAs are largely imported from the blood to the liver against concentration gradient via protein-mediated transport (Cusi 2012). Protein-mediated hepatic LCFAs uptake is facilitated by a number of transport proteins like fatty acid-binding protein (FABPpm), fatty acid transport proteins (FATP 2 and 5) and fatty acid translocase (FAT/CD36) (Glatz et al. 2010). Moreover, lipid efflux from the liver is also to a great extent protein dependent, involving microsomal triacylglycerol transport protein (MTP) and cassette transporter (ABCA1) (Holthuis & Levine 2005, Parks et al. 2012). Nonetheless, the role of these transporters in hepatocytes, especially in the context of nonalcoholic fatty liver disease development and the influence of dexamethasone on its function, is still not clear.

This study sought to determine whether dexamethasone, in the presence of increased palmitic acid availability, would influence intrahepatic lipid accumulation, fatty acid composition and sphingolipid concentration, which most likely affect NAFLD development. We hypothesized that DEX would not only modulate protein-mediated fatty acid uptake but also exert an effect on lipid secretion out of the liver cells, which may be important for understanding alterations in lipid metabolism after glucocorticoid treatment.

Materials and methods

Primary rat hepatocytes isolation
Male Wistar rats (body mass 220–250 g) were maintained at 22°C ± 2 on a reversed light-darkness cycle in approved animal holding facilities with unrestricted access to water and standard chow for rodents (Agropol, Motycz, Poland). The experiments were carried out after one week of acclimatization of the animals. All the procedures and the number of animals were approved by the Ethics Committee on Animal Care at the Medical University of Białystok. Isolation of primary rat hepatocytes from rat’s liver was performed using collagenase perfusion system according to the Seglen’s method (Seglen 1976). The procedure of rat liver perfusion with minor modifications was described in detail previously (Konstantynowicz-Nowicka et al. 2015). After perfusion, hepatocytes were seeded in 12-well collagen-coated plates and incubated for 24 h in DMEM (Immuniq, Zory, Poland) supplemented with 10% fetal bovine serum (FBS, Polgen) and 1% antibiotic/antimycotic (penicillin/streptomycin, Immuniq) at 37°C in a humidified atmosphere (5% CO₂). The attached cells were washed twice with PBS (Immuniq), and their morphology and viability were assessed in Bürker chamber using Trypan blue (Sigma-Aldrich) staining during all the incubation periods. Each experiment was always carried out on 10⁶ cells per well and the percentage of living cells was above 85%.

Palmitate and dexamethasone treatment
Primary rat hepatocytes were incubated in the presence or absence of palmitic acid (PA) and dexamethasone (DEX) at the concentration of 0.75 mM and 1 µM, respectively, for 16h and 40h. Palmitic acid before adding to the
incubation media was conjugated with fatty acid-free bovine serum albumin (BSA), as it was described in detail previously (Konstantynowicz-Nowicka et al. 2015). At the end of each incubation period, samples of the medium were taken and immediately frozen in the temperature of liquid nitrogen. Moreover, cells were homogenized in ice cold RIPA lysis buffer containing protease and phosphatase inhibitors (Roche Diagnostics GmbH). Before further analysis, the protein concentration was determined in all the samples using the bicinchoninic acid method (BCA) with fatty acid-free bovine serum albumin as a standard.

**Intracellular and extracellular lipid analyses**

Primary rat hepatocytes at the end of each incubation period were suspended in ice cold phosphate buffered saline (PBS) and immediately frozen in the temperature of liquid nitrogen. Thereafter, the cells were homogenized by ultrasonication, and protein concentration in each sample was measured. Additionally, we analyzed fatty acid composition of incubation media at the end of each set of the experiment. As we described previously (Chabowski et al. 2013) lipids from primary hepatocytes and medium were extracted in a chloroform–methanol solution according to the Folch method (Folch et al. 1957). Briefly, the diacylglycerol (DAG) and triacylglycerol (TAG) fractions were separated by using thin-layer chromatography (TLC) (van der Vusse et al. 1980). Individual fatty acid methyl esters were identified and quantified according to the retention times of standards by GLC (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column). Total DAG and TAG contents were estimated as the sum of particular fatty acid species of the assessed fractions, and it was expressed in nanomoles per the amount of protein in each sample. Moreover, based on unsaturated (USFA) and saturated (SFA) fatty acid compositions, we checked the fatty acid saturation status regardless of the total lipid content in the examined lipid fractions (desaturation index).

**Intracellular and extracellular sphingolipid analyses**

The contents of ceramide, sphinganine and sphingosine were determined as described previously in detail by Baranowski et al. (2008). Briefly, all the samples, apart from incubation media, were homogenized by ultrasonication and lipids were extracted in the presence of internal standard (10 pmol of C17-sphingosine, Avanti Polar Lipids). An aliquot of the lipid extract was transferred to a fresh tube with pre-added 40 pmol of N-palmitoyl-δ-erythro-sphingosine (C17 base) as an internal standard and then subjected to alkaline hydrolysis to deacylate ceramide to sphingosine. The free sphinganine, as well as sphingosine released from ceramide were then converted to their o-phthalaldehyde derivatives and analyzed using a HPLC system. Before the sphingolipid analysis, the protein content was measured.

**Immunoblotting analyses**

Routine Western blot method was used to show the expression of selected proteins involved in fatty acid transport and lipid metabolism in hepatocytes. As we described previously (Konstantynowicz-Nowicka et al. 2015), after SDS polyacrylamide gel electrophoresis, transfer and blocking (5% of nonfat dry milk or BSA), the membranes were incubated with primary antibodies FAT/CD36, FABPpm (Abcam), FATP-2, FATP-5, MTP, GAPDH (Santa Cruz Biotechnology) and ABCA1 (Thermo Scientific). Thereafter, nitrocellulose membranes were incubated with appropriate secondary antibody labeled with horseradish peroxidase (Santa Cruz Biotechnology). Signals obtained by immunoblotting were quantified densitometrically using a ChemiDoc visualization system (Bio Rad). Equal concentration of protein was loaded on each line (30 µg), which was confirmed by Ponceau S staining. Moreover, the protein expression was standardized to the intracellular expression of GAPDH and the control was set as 100%.

**Statistical analyses**

All the data are expressed as mean values ± S.D. based on six independent determinations. Statistical difference between groups was tested with two-way analyses of variance (ANOVA) and appropriate post hoc tests using STATISTICA version 10 (StatSoft, Krakow, Poland). Results were considered to be statistically significant at $P < 0.05$.

**Results**

**Effects of 16-h and 40-h incubation of primary hepatocytes with PA and/or DEX on the intracellular contents of TAG and DAG fractions**

In primary rat hepatocytes, both short and prolonged incubations with DEX as well as palmitate resulted in significant accumulation of intracellular TAGs, in all the examined groups compared with the respective control.
group (16 h, PA: +287.8%, DEX: +58.5%, DEX+PA: +215.1%, P < 0.05; 40 h, PA: +310.7%, DEX: +37.6%, DEX+PA: +326.6%, P < 0.05; Fig. 1A). Furthermore, PA alone increased TAG content in primary hepatocytes after 16-h incubation (P < 0.05, Fig. 1A), but subsequently it was reduced by DEX (16 h, DEX+PA: −72.7%, P < 0.05; Fig. 1A; vs PA group). Interestingly, DAG fraction after 16-h incubation of primary hepatocytes remained unchanged, whereas prolonged treatment resulted in significant reduction in all the examined groups compared with the control group (40 h, PA: −17.4%, DEX: −39.2, DEX+PA: −27.8%, P < 0.05; Fig. 1C).

Effects of 16-h and 40-h incubation of primary hepatocytes with PA and/or DEX on the extracellular contents of TAG and DAG fractions

As expected, the extracellular TAG content in primary rat hepatocytes was significantly elevated in all the examined groups after short (16 h, PA: +95.6, DEX: +54.6%, DEX+PA: +295.9%, P < 0.05; Fig. 1B) as well as prolonged (40 h, PA: +48.9%, DEX: +94.7%, DEX+PA: +351.5%, P < 0.05; Fig. 1B) incubation period in comparison with respective control group. Importantly, DEX induced further increase in the extracellular TAG level in the PA-treated groups in each experimental time (16 h, DEX+PA: +200.3%, P < 0.05; 40 h, DEX+PA: +302.6%, P < 0.05; Fig. 1B; vs PA groups). Similarly, the extracellular DAG content in primary hepatocytes incubation media was increased either by PA or DEX at the end of short (16 h, DEX: +144.1%, DEX+PA: +186.9%, P < 0.05; Fig. 1D) and prolonged (40 h, PA: +77.5%, DEX: +165.9%, DEX+PA: +143.3%, P < 0.05; Fig. 1D) treatment compared with a proper control group except for the PA group after 16-h incubation. Moreover, the extracellular DAG levels were even further increased by DEX in all the PA-treated groups similar to the extracellular TAG content (16 h, DEX+PA: +175.5%, P < 0.05; 40 h, DEX+PA: +65.8%, P < 0.05; Fig. 1D; vs PA groups).

Effects of 16-h and 40-h incubation of primary hepatocytes with PA and/or DEX on the intracellular fatty acid composition in TAG and DAG fractions

As expected, composition of the saturated (C16:0, C18:0) and unsaturated (C16:1, C18:1) fatty acids was significantly elevated in the intracellular TAG fraction by PA and/or DEX treatment compared with that in the control primary rat hepatocytes irrespective of the incubation time (P < 0.05; Fig. 2A). Moreover, short-term exposure of primary hepatocytes to PA and DEX simultaneously resulted in a decline in the content of examined fatty acids in the TAG fraction, but the drop in C16:1 and C18:0 was significant compared with the respective PA group (P < 0.05; Fig. 2A). Unexpectedly, there was a trend toward reduction in the saturated and unsaturated fatty acids content in the intracellular DAG fraction, especially at the end of 40-h incubation (Fig. 2C). The contents of C16:0, C16:1, C18:1 (40 h) and C18:0 (16 h and 40 h) were considerably reduced compared with those of corresponding control groups (P < 0.05; Fig. 2C) as the effect of PA and/or DEX exposure. However, after short treatment, PA alone or with DEX induced significant accumulation of C16:0,
C16:1 and C18:1 in DAG fraction in primary rat hepatocytes compared with respective control group ($P < 0.05$; Fig. 2C).

**Effects of 16-h and 40-h incubation of primary hepatocytes with PA and/or DEX on the extracellular fatty acid composition in TAG and DAG fractions**

Intracellular accumulation of examined fatty acids in TAG fraction in primary rat hepatocytes resulted in a significant secretion of C16:0, C16:1, C18:0 and C18:1 into incubation media in all the examined groups compared with the respective control group after 16h ($P < 0.05$; Fig. 2B). Moreover, extracellular accumulation of C16:1, C18:0 and C18:1 in TAG fraction was further increased by DEX in PA-treated groups at the end of 16-h incubation period and C16:0, C16:1, C18:0 and C18:1 after 40-h incubation period in primary hepatocytes ($P < 0.05$; Fig. 2B; vs respective PA group). However, the long-term experiment revealed a significant secretion of C16:0 into incubation media in TAG fraction in all the examined groups and C16:1, C18:0 and C18:1 in groups treated with DEX alone or with PA compared with a proper control group in primary hepatocytes ($P < 0.05$; Fig. 2B). Substantial secretion of saturated as well as unsaturated fatty acids in DAG fraction in primary rat hepatocytes was a result of decreases in the intracellular fatty acids content. We observed a considerable accumulation of C16:0, C16:1, C18:0 and C18:1 in DAG fraction in incubation media caused by PA and/or DEX treatment compared with proper control group irrespective of exposure time ($P < 0.05$; Fig. 2D). However, after 40-h incubation,
PA alone or together with DEX markedly decreased extracellular content of C16:1 and C18:1 in DAG fraction in primary rat hepatocytes compared with the control group, respectively ($P<0.05$; Fig. 2D). Moreover, each examined fatty acid was significantly increased by DEX in PA-treated groups (16-h incubation), simultaneously DEX decreased the extracellular content of C16:0 and C18:1 in the same groups (40-h incubation), compared with respective PA group ($P<0.05$; Fig. 2D).

**Effects of 16-h and 40-h incubation of primary hepatocytes with PA and/or DEX on saturation status of TAG and DAG fractions**

The saturation status of primary hepatocytes in TAG fraction was significantly affected. There was a drop in 16:1/16:0 ratio at the end of short as well as prolonged treatment in PA and PA+DEX groups compared with the respective control cells (16h, PA: −58.4%, PA+DEX: −61.7%, $P<0.05$; 40h, PA: −65.2%, DEX+PA: −64.9%, $P<0.05$; Fig. 3A). Accordingly, the 18:1/18:0 ratio in TAG fraction was also markedly decreased in the same primary hepatocytes in each treatment time (16h, PA: −31.9%, PA+DEX: −24.9%, $P<0.05$; 40h, PA: −21.2%, DEX+PA: −24.1%, $P<0.05$; Fig. 3B, vs respective control group). Interestingly, the desaturation index (16:1/16:0 ratio) in DAG fraction was considerably elevated to the same extent in all the examined groups after 40h and in DEX+PA-treated primary hepatocytes after 16h (16h, DEX+PA: +36.3%, $P<0.05$; 40h, PA: +57.8%, DEX: +44.6%, DEX+PA: +69.7%, $P<0.05$; Fig. 3C, vs respective control group). Simultaneously, PA as well as DEX alone or together significantly increased 18:1/18:0 ratio in comparison with the corresponding control group in DAG fraction in primary hepatocytes (16h, PA: +132.5%, DEX: +56.6%, DEX+PA: +67.7%, $P<0.05$; 40h, PA: +100.1%, DEX: +22.1%, DEX+PA: +24.6%, $P<0.05$; Fig. 3D).

Additionally, the cells treated with DEX combined with PA had considerably reduced 18:1/18:0 ratio in DAG fraction compared with the PA alone (16h, DEX+PA: −27.8%, $P<0.05$; 40h, DEX+PA: −37.7%, $P<0.05$; Fig. 3D).

**Effects of 16-h and 40-h incubation of primary rat hepatocytes with PA and/or DEX on total expression of proteins involved in lipid metabolism**

There was a significant increase in the expression of SCD1 in primary rat hepatocytes after 16-h PA and/or DEX treatment compared with the control group ($P<0.05$; Fig. 4A). Moreover, the expression of SCD1 in DEX+PA group vs Palmitate group; **P**<0.05 significant difference vs control group; **P**<0.05 significant difference: Dexamethasone+Palmitate group vs Palmitate group; **P**<0.05 significant difference: Dexamethasone+Palmitate. (16h, PA: +24.4%, DEX+PA: +27.6%, $P<0.05$; Fig. 4A), which was even more pronounced after 40h (PA: +51.9%, DEX: +69.1%, DEX+PA: +91.7%, $P<0.05$; Fig. 4A). On the other hand, the CPT I expression remained unchanged in all the examined groups regardless of the incubation time ($P<0.05$, Fig. 4B).

**Effects of 16-h and 40-h incubation of primary rat hepatocytes with PA and/or DEX on intracellular sphingolipid content**

Primary rat hepatocytes exposed to PA and/or DEX (16h and 40h) significantly increased the intracellular content of sphinganine compared with respective control group (16h, PA: +22.6%, DEX: +31.1%, DEX+PA: +65.9%, $P<0.05$; 40h, PA: +144.4%, DEX: +101.9%, DEX+PA: +147.1%,
P < 0.05; Fig. 5B). Moreover, intensified de novo ceramide synthesis was also confirmed by a considerable accumulation of the sphingolipid in all the examined groups irrespective of incubation time (16 h, PA: +49.8%, DEX: +29.3%, DEX + PA: +52.5%, P < 0.05; 40 h, PA: +99.1%, DEX: +45.5%, DEX + PA: +82.7%, P < 0.05; Fig. 5A) compared with the control group. Similar to the other sphingolipids, intracellular sphingosine levels were markedly elevated by PA and/or DEX, especially after short-term incubation (16 h, PA: +63.8%, DEX: +47.4%, DEX + PA: +51.1%, P < 0.05; 40 h, DEX: +72.8%, DEX + PA: +29.9%, P < 0.05; Fig. 5C) compared with those in the control.

Effects of 16-h and 40-h incubation of primary rat hepatocytes with PA and/or DEX on extracellular sphingolipid content

As expected, each incubation time revealed a significant accumulation of ceramide in culture media in all the examined primary rat hepatocytes compared with the corresponding control group (16 h, PA: +276.1%, DEX: +136.7%, DEX + PA: +115.9%, P < 0.05; 40 h, PA: +163.6%, DEX: +55.6%, DEX + PA: +96.5%, P < 0.05; Fig. 5D). However, irrespective of incubation time, DEX in PA-treated primary hepatocytes markedly decreased the secretion of ceramide into media (16 h, DEX + PA: −25.5%, P < 0.05; Fig. 5D, vs respective PA groups). Opposite effects of PA and/or DEX treatment were observed in extracellular sphingosine content. At the end of both incubation periods, there was a significant decline in all the examined primary hepatocytes compared with the control groups (16 h, PA: −33.3%, DEX: −18.7%, DEX + PA: −36.9%, P < 0.05; 40 h, PA: −64.5%, DEX: +32.3%, DEX + PA: −50.2%, P < 0.05; Fig. 5E) apart from 40-h DEX treatment, which was markedly increased.

Effects of 16-h and 40-h incubation of primary rat hepatocytes with PA and/or DEX on total expression of proteins involved in plasmalemmal, intracellular and extracellular lipid transport

Total expression of FAT/CD36 (16 h, DEX: +37.4, DEX + PA: +33.1, P < 0.05; Fig. 6A) as well as FABPpm (40 h, DEX: +23.1%, DEX + PA: +31.8%, P < 0.05; Fig. 6B) in primary rat hepatocytes was significantly increased only by DEX alone or with PA after short and prolonged incubation time compared with the control groups, respectively. Elevation of FATP2 (16 h, PA: +38.3%, DEX: +50.5%, DEX + PA: +37.3%, P < 0.05; 40 h, PA: +45.8%, DEX: +27.9%, DEX + PA: +49.9%, P < 0.05; Fig. 6C) and FATP5 expression (16 h, PA: +24.9%, DEX: +22.1%, DEX + PA: +23.3%, P < 0.05; 40 h, PA: +70.9%, DEX: +54.4%,
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DEX + PA: +41.1%, P < 0.05; Fig. 6D) was even more pronounced. There was a considerable rise in the expression of these proteins in all the examined primary rat hepatocytes compared with the corresponding control groups irrespective of incubation time. As expected, PA and/or DEX induced a significant increase in MTP (16 h, PA: +36.9%, DEX: +55.4%, DEX + PA: +76.1%, P < 0.05; 40 h, PA: +77.6%, DEX: +77.7%, DEX + PA: +97.4%, P < 0.05; Fig. 6E) as well as ABCA1 (16 h, PA: +30.2%, DEX: +43.4%, DEX + PA: +21.1%, P < 0.05; 40 h, PA: +70.6%, DEX: +94.1%, DEX + PA: +64.7%, P < 0.05; Fig. 6F) expression compared with the respective control cells after short and prolonged experimental time. Furthermore, DEX in palmitate-treated primary hepatocytes additively increased MTP expression after both experimental times compared with the PA alone, but only short-term treatment resulted in a significant elevation (DEX + PA: +39.1%, P < 0.05; Fig. 6E).

Discussion

The short and prolonged incubation was carried out to investigate the time-dependent effect of dexamethasone alone and in combination with palmitate on saturation status, lipid content and transport, which are extremely important issues in liver pathology to elucidate.

The experiments were conducted on isolated primary rat hepatocytes representing normal mature hepatocytes.

It is well known that dexamethasone induces TAG accumulation in liver (Dich et al. 1983, Mangiapane & Brindley 1986, Wang et al. 2012). In our studies, dexamethasone treatment also resulted in increased TAG deposition. This is not surprising, as used incubation media was rich in glucose and sodium pyruvate, which are a good source for de novo lipogenesis. Studies conducted by other researchers have shown that glucocorticoids are potent regulators of major lipogenic enzymes activity like fatty acid synthase (Tomita et al. 1993, Giudetti & Gnoni 1998). Moreover, another source of TAG accumulation in dexamethasone group was probably re-esterification from different lipid fractions, e.g., free fatty acids, as it was indicated that glucocorticoids stimulate the expression of genes taking part in free fatty acid re-esterification also (Dolinsky et al. 2004). It was shown in previous studies, and it was confirmed in our settings, as the treatment of primary hepatocytes with high dose of palmitate resulted in increased accumulation of triacylglycerols, which is thought to be a primary determinant of NAFLD (Budick-Harmelin et al. 2012). However, additional treatment with dexamethasone either decreased (after 16-h incubation) or had no significant additive effect (prolonged exposure) on intrahepatocellular TAG content. Simultaneously,
a significant decrease in DAG lipid pool was observed. Partly in line with our results are studies conducted on rats fed with high-fat diet and exposed to chronic stress, where decreased liver triacylglycerol and cholesterol contents were observed (Han et al. 2015). However, in this model, chronic stress caused elevation not only in cortisol but also adrenaline and growth hormone concentrations in rats’ serum, which may also affect liver lipid deposits. Moreover, in this study, DEX has changed TAG concentration after 40-h incubation in comparison with control group in the examined cells. What is extremely important is that our studies imply that DEX with high-fat diet may alter hepatocytes lipid metabolism in a time-dependent manner, which explains why some researchers found different DEX effects on TAG accumulation in liver using different times of exposition (Han et al. 2015, Hubel et al. 2015, Kumar et al. 2015).

Interestingly, we observed that combined treatment (DEX with palmitate) additively increased the expression of selected examined fatty acid transporters (FAT/CD36 16h and FABPpm 40h) in primary rat hepatocytes. This may imply that fatty acid uptake was increased and subsequently reflected by increased intracellular TAG deposition. In contrast, a report from D’Souza and coworkers showed that after corticosterone treatment combined with high-fat diet administration, alterations were observed only in liver FAT/CD36 expression, but not FABPpm (D’Souza et al. 2012). One possible explanation for this discrepancy is that the aforementioned researchers analyzed the total liver homogenates, which consist of different hepatic cell types such as Kupffer cells and macrophages, which greatly express FAT/CD36, compared with the hepatocytes cell cultures used in our studies.

Importantly, hepatocytes are able to secrete lipids in the process, which is facilitated by proteins such as MTP and ABCA1. The aforementioned proteins are highly involved in the export of lipids and play an important regulatory role in intrahepatocellular accumulation of lipids also (Holthuis & Levine 2005, Parks et al. 2012). In primary rat hepatocytes, in the presence of high availability of palmitate, dexamethasone led to not only increased intracellular TAG deposition but also significantly elevated transport of this fraction out of the cell. There are few and somewhat contradictory data on the influence of dexamethasone on lipids secretion out of hepatocytes. Wang and coworkers, in human studies, found that dexamethasone did not exert any significant effect on the secretion of VLDL rich in TAGs, and concomitantly, plasma-free fatty acids concentration was not increased (Wang et al. 2012b). The discrepancies between these (in vivo) and our studies (in vitro) may be explained by a different experimental model. In contrast, others revealed that dexamethasone increased hepatic TAG secretion as well as its plasma level, which is in accordance with our findings (Sivabalasubramanian et al. 2008, Han et al. 2015). What is most interesting is that dexamethasone together with palmitate exerted additive effect on increased TAG secretion during short and prolonged incubation. Taking these data into account, our present findings show that primary hepatocytes export TAG and DAG to the incubation media, which suggests that extracellular fatty acid transport may be an efficient protective mechanism of the liver. However, in our settings, the influx of fatty acids was still overwhelmed and accumulation of TAG occurred. To further address this issue, we investigated the influence of dexamethasone on protein transporters expression, involved in extracellular transport of lipids, which may elucidate possible uncertainties in the role of these proteins in the development of NAFLD. There are some studies showing strong influence of dexamethasone on extracellular transporters in liver (Sporstol et al. 2007, Han et al. 2015). Among them, there are the results suggesting that dexamethasone increases the mRNA expression of ABCA1 in primary rat hepatocytes with no changes in HepG2 expression (Sporstol et al. 2007). Moreover, in the studies in which rats were fed a high-fat diet and exposed to chronic stress, the expression of MTP transporter was markedly increased (Han et al. 2015). This is in line with the data presented herein, where synthetic glucocorticoid and palmitate showed additive effect in increasing hepatic MTP expression. However, the additive effect was not confirmed with respect to the expression of ABCA1 although the ABCA1 expression was also increased after separate treatments in comparison with the control group. The observed changes in hepatic MTP and ABCA1 expression are in accordence with elevated extracellular TAG and DAG concentrations. Thus, the additive effect noticed in MTP expression may be the evidence that dexamethasone, together with palmitate, alters fatty acid efflux from hepatocytes.

In our in vitro model, palmitic acid induced the accumulation of not only di- and triacylglycerols, but also other lipid fractions such as sphingolipids. There are in vivo studies in which increased accumulation of ceramide in the liver was the result of corticosterone administration in high-fat fed rats (D’Souza et al. 2012). However, our observations also indicated that dexamethasone and palmitate treatments led to the induction of ceramide
biosynthesis pathway with an elevated concentration of ceramide and sphinganine in primary hepatocytes. Other studies conducted on high-fat fed rats administering dexamethasone showed an increased accumulation of ceramide in the liver and skeletal muscles, by inducing serine palmitoyltransferase 2 expression required for sphingolipid de novo synthesis (Holland et al. 2007). In the absence of substrates used for de novo synthesis, as it was observed in our dexamethasone group, ceramides may be synthesized from free fatty acid re-esterification also. Moreover, we cannot exclude the possibility that another source of ceramides in this experimental group is sphingomyelin hydrolysis as it was documented in HeLa cells (Johnston et al. 1980). Furthermore, it was observed in our study that increased deposition of sphingolipid fractions, which are thought to be highly bioactive lipid compounds, may lead to insulin resistance development during NAFLD, and it can be a risk factor for its deterioration as it was shown by prior studies in skeletal muscle and liver (Holland et al. 2007, D’souza et al. 2012).

Our previous studies indicated that hepatocytes, during increased bioavailability of palmitate, were able to not only excessively accumulate sphingolipids but also transport them out of the cell (Konstynownicz-Nowicka et al. 2015). The effect of dexamethasone treatment on export of sphingosine-1-phosphate was merely investigated in cultured fibroblasts, where a significant increase was observed (Nieuwenhuis et al. 2009). Nevertheless, we are not aware of any studies that have examined the effect of DEX on hepatic sphingolipids transport out of the cells. These findings have demonstrated for the first time that the main sphingolipid fraction found in post-incubation media after DEX treatment is ceramide. Surprisingly, dexamethasone combined with palmitate decreased ceramide concentration in the incubation media in primary hepatocytes after 16 and 40h comparing with palmitate alone.

Although the lipid saturation status has been confirmed to affect the accumulation rate of lipid fractions (Timmers et al. 2011), this is the first report that describes the role of dexamethasone on hepatocytes desaturation ratio during increased availability of saturated fatty acid. Stearoyl-CoA desaturase (SCD1) is thought to be the most important enzyme that catalyzes the rate-limiting step in the synthesis of triglycerides and cholesterol esters that converts saturated to unsaturated fatty acids, mainly 16:0 and 18:0 (Paton & Ntambi 2009, Fernandez et al. 2011). To the best of our knowledge, there is only one previous report where dexamethasone increased SCD1 activity in chickens skeletal muscles as well as in primary cultured myoblasts, but only in fasted state with no changes after feeding (Wang et al. 2012c). By contrast, in our studies, we observed increased expression of SCD1 after 16-h and 40-h incubation with palmitate and/ or DEX in primary hepatocytes. To definitively address this issue, we investigated TAG and DAG desaturation index that is a simple SCD1 activity indicator (Attie et al. 2002, Jeyakumar et al. 2009). We demonstrated that decreased TAG desaturation ratio especially after 40h is consistent with elevated accumulation of saturated rather than unsaturated fatty acids in primary rat hepatocytes. Consequently, SFAs in triacylglycerol fraction are not preferably used as substrates in fatty acid synthesis. This is not surprising as hepatocytes will most likely not augment de novo lipid synthesis when its intracellular lipid depots are excessive, which may affect proper cell functioning. However, in DAG fraction, we observed a significant increase in desaturation index after DEX treatment with elevated unsaturated fatty acid accumulation. This may result from the increased bioavailability of substrates used in synthesis/esterification of other lipid fractions such as TAG and probably may lead to alteration in fatty acid composition as well as its redistribution between lipid pools. The results reported by Alaniz and coworkers showed increased SCD1 activity with increased unsaturated fatty acid composition after dexamethasone treatment in rat liver microsomes (de Alaniz & Marra 2003). It is only in part in accordance with our studies that show discrepancies in desaturation index and SCD1 expression. One possible explanation for these differences is that we assessed lipid composition and protein expression in whole cell homogenates rather than liver microsomes. Even though we observed an increase in SCD1 expression in abundance of palmitate, it was not sufficient to produce C16:1 and C18:1 fatty acid in TAG fraction. The observed changes are even more pronounced after prolonged incubation, which indicates a direction in metabolic routes. For the liver, it is more likely to accumulate triacylglycerols, which are subsequently the source of FA esterified to various lipid fractions or secreted, rather than deposit diacylglycerols that may impair cell signaling pathways (such as insulin). In our study, DAG accumulation was not observed in primary hepatocytes and probably the effectiveness of SCD1 was much greater, which resulted in increased desaturation ratio. This could be beneficial as TAG accumulation leads to hepatic steatosis. However, it is safer than deposition of other, more biologically active fractions like DAG or sphingolipids.
According to Timmers and coworkers, skeletal muscle DAG and TAG composition is consistent with a variety of fatty acids present in diet (Timmers et al. 2011). Supporting this notion, we also found that increased concentration of palmitic acid in incubation media resulted mainly in elevated proportion of this fatty acid in TAG fraction with lesser, but still significant accumulation of other lipid fractions. After short incubation, dexamethasone slightly decreased the deposition of both saturated and unsaturated fatty acids in comparison with palmitate group. However, the decreased accumulation of DAGs in primary hepatocytes is consistent with reduced SFA as well as USFA composition. This may be advantageous as others have hypothesized that DAG derived from saturated fatty acids is a less biologically active molecule, which does not affect insulin signaling (Holland et al. 2007).

The data presented herein also showed that dexamethasone treatment together with palmitate significantly increased the export of all the examined fatty acids. These findings suggest that in rat hepatocytes, dexamethasone induced both redistribution of intracellular fatty acids in DAG toward TAG fraction as well as intensified export of excessive lipids. Fatty acid transport out of the cell induced by dexamethasone is advantageous as others have suggested that DAG derived from saturated fatty acids are protected to some extent against increased deposition of FA in different lipids.

Nonetheless, fatty acid export is not the only possibility of hepatocytes to protect themselves against lipid overload. The fatty acid accumulation may occur when cells’ β-oxidation capacity is overwhelmed (Hautekeete et al. 1990, El-Badry et al. 2007). In some in vivo studies, β-oxidation was shown to be upregulated during high-fat diet with no changes after liver’s exposure to chronic stress (Han et al. 2015). However, in experiments conducted on muscle cells, dexamethasone diminished palmitate oxidation (Wang et al. 2012c). In primary rat hepatocytes, we did not observe changes in carnitine palmitoyltransferase I (CPT I) expression, which shows that hepatocytes may export fatty acids out of the cell rather than oxidize them like in skeletal muscles. In primary hepatocytes, we observed only a trend toward an increase in CPT I expression with the opposite effects after 40-h incubation. This would suggest that for hepatocytes, lipid redistribution among different fatty acid fractions, incorporation to lipoproteins and transport out of the cell play a pivotal role against lipid accumulation. It is possible that this mechanism is more effective than oxidation, especially during constantly elevated palmitate availability and increased fatty acid uptake. Moreover, amplified fatty acid concentration in incubation media may deteriorate mitochondrial functioning, which can probably lead to elevated oxidative stress that affects cell homeostasis.

In conclusion, our study indicates that dexamethasone combined with palmitate stimulated protein-facilitated fatty acid uptake in primary hepatocytes. This resulted in increased accumulation of TAGs and sphingolipids with concomitant alterations in fatty acid composition. Dexamethasone combined with high availability of fatty acids decreased TAG desaturation index, which was a sign of intracellular lipids redistribution with possibly repressed β-oxidation. These findings provide evidence that dexamethasone with elevated fatty acid concentration not only affects lipid profile in hepatocytes but also exerts stimulating additive effect on, mediated by MTP and ABCA1, lipid export. Affected by glucocorticoids, lipid transport may be an important protective mechanism against lipotoxicity and deterioration of NAFLD.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
E H S and K K N were responsible for the conception and design of the study, analysis and interpretation of data, design of the article and drafting the article. A C was responsible for the conception and design of the study, analysis and interpretation of data and revised the manuscript. All authors gave final approval.

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