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Hepatic Stellate Cell Activation: A Source for Bioactive Lipids

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Abstract

Hepatic stellate cells (HSCs) are non-parenchymal liver cells that characteristically contain multiple retinoid (vitamin A)-containing lipid droplets. In this study, we addressed the metabolic fate of non-retinoid lipids originating from lipid droplet loss during HSCs activation. UPLC/MS/MS and qRT-PCR were used to monitor the lipid composition and mRNA expression of selected genes regulating lipid metabolism in freshly isolated, overnight-, 3- and 7-day cultures or primary mouse HSCs. A preferential accumulation of specific C20-C24 fatty acid species, especially arachidonic (C20:4) and docosahexaenoic acids (C22:6), was revealed in culture-activated HSCs along with an upregulation of transcription of fatty acid desaturases (*Scd1*, *Scd2*) and elongases (*Elovl5*, *Elovl6*). This was accompanied with an enrichment of activated HSCs with 36:4 and 38:4 phosphatidylcholine species containing polyunsaturated fatty acids and associated accumulation of selective lipid mediators, including endocannabinoids and related N-acyl ethanolamides, as well as ceramides. An increase in 2-arachidonoylglycerol and N-arachidonoyl ethanolamide concentrations was observed along with an upregulation of *Dagla* mRNA expression in HSCs during culture activation. N-palmitoyl ethanolamide was identified as the most abundant endocannabinoid-like species in activated HSCs. An increase in total ceramide levels and enrichment with N-palmitoyl (C16:0), N-tetracosenoyl (C24:1), N-tetracosanoyl (C24:0) and N-docosanoyl (C22:0) ceramides was detected in activated HSC cultures and was preceded by increased mRNA expression of ceramide synthesizing enzymes (*CerS2*, *CerS5* and *Smpd1*). Our data suggest an active redistribution of non-retinoid lipids in HSCs underlying the formation of low abundance, highly bioactive lipid species that may affect signaling during HSC activation, as well as extracellularly within the liver.

Graphical Abstract

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Keywords

Lipid droplets; polyunsaturated fatty acids; phosphatidylcholine; endocannabinoids; ceramides; lipidomics

1. INTRODUCTION

Hepatic stellate cells (HSCs) are non-parenchymal liver cells (also called perisinusoidal cells, Ito cells, lipocytes or fat-storing cells) residing in the space of Disse and constituting about 8 % of the total cells present in a healthy liver. HSCs are characterized by their abundant highly specialized lipid droplets, which represent the subcellular site of storage of the majority of the liver's retinoid (vitamin A and its natural metabolites) reserves [1]. Along with retinoids, HSC lipid droplet lipid composition comprises other non-retinoid lipids including triglycerides, cholesterol and its esters, free (unesterified) fatty acids and different phospholipid species [1, 2]. Upon activation, observed *in vivo* during liver injury or *in vitro* upon culture of primary cells on plastic, HSCs rapidly lose their characteristic lipid droplets and extensively produce extracellular matrix components (i.e. α -smooth muscle actin, desmin, type I and III collagens and fibronectin) and profibrogenic cytokines (TGF β 1) [3]. The retinoids liberated from lipid droplets during HSC activation can be used for retinoic acid formation, affecting the optimal transcription of retinoid-responsive genes [4]. However, the metabolic roles and fates of the non-retinoid lipids present in lipid droplets during HSC activation remain unclear. To address this, qRT-PCR and UPLC/MS/MS were used to assess possible changes in the lipid composition of plastic-activated freshly isolated mouse HSCs after overnight-, 3- and 7-days of culturing.

2. MATERIALS AND METHODS

2.1. Animal husbandry

All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of Columbia University according to criteria outlined in the Guide for

the Care and Use of Laboratory Animals prepared by the National Academy of Sciences [5]. Ten- to twelve-week-old male C57BL/6J mice were used for all studies.

2.2. Isolation of primary hepatic stellate cells

Mice were anesthetized with a cocktail consisting of 1 mL Ketaset (100 mg/ml), 0.5 mL xylazine (100 mg/ml), and 8.5 mL of phosphate buffered saline (PBS) given at a dose of 10 μ L/g body weight. The abdomen was opened to allow access to the liver. With the organs pulled to the side, a catheter was angled parallel to the mouse's body and was inserted into the vein that connects the kidney to the inferior vena cava. Primary HSCs were isolated by *in situ* liver perfusion with a Gey's balanced salt solution (GBSS) containing Pronase E (EMD Chemicals Inc., Gibbstown, NJ) and collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ). The liver was first perfused at a flow rate of 5 ml/min with a solution containing EGTA, followed by successive perfusions with GBSS containing Pronase E (0.4 mg/ml) and collagenase IV (0.5 mg/ml), for 5 min and 8 min, respectively. Immediately after the first solution entered the liver, the portal vein was cut and the suprahepatic inferior vena cava was clamped. After perfusion, the partially digested liver was excised, the digest passed through a 100 μ m nylon mesh to remove undigested materials, and resuspended in GBSS containing 0.5 mg/ml Pronase E, 0.5 mg/ml collagenase IV and 2 mg/ml DNase I (Milipore Sigma, USA). The suspension was incubated in a beaker placed on a stirring platform for 25 min at 37 °C. HSCs were purified from the remainder of non-parenchymal cells and hepatocyte-derived debris by floatation through 9% (w/v) Nycodenz (Accurate Chemical & Scientific Corp., Westbury, NY) in GBSS. The average yield of cells, determined by counting using a hemocytometer, was $1.18 \pm 0.32 \times 10^6$ cells per mouse liver.

After isolation, a fraction of the cells was either collected for analysis (referred to as freshly isolated or day 0 cells) or plated in 6-well plastic plates at a density 2×10^5 cells/well followed by culture in DMEM (Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Peak Serum, Wellington, CO, USA), 100 units/ml penicillin and 100 μ g/ml streptomycin (Life Technologies Corporation, Carlsbad, CA, USA) in a humidified 5% CO₂ incubator at 37 °C. Culture medium was first changed after overnight culture and subsequently every 2 days. Images of HSCs at different days in culture were captured at $\times 20$ and $\times 40$ magnifications using Olympus FSX100 microscope (Center Valley, PA, USA).

For the analyses, attached cells were washed with PBS, treated with 0.25% trypsin-EDTA solution (Life Technologies Corporation, Carlsbad, CA, USA) and then collected. Cells were harvested after overnight culture (day 0.5 time point), and 3- and 7-days culture (day 3 and day 7 time points respectively). Collected cells were pelleted and snap frozen in liquid nitrogen and stored at -80 °C until analysis. The DNA concentration of each cell sample was assessed employing High Sensitivity Quant-iT™ dsDNA Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to manufacturer's protocol.

2.3. RNA preparation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from frozen cell pellets employing TRIzol Reagent (Ambion, Foster City, CA, USA) and isolated using the E.Z.N.A Total RNA Kit II (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. RNA was quantitated at 260 nm using a Nanodrop spectrophotometer. cDNA synthesis was performed using 0.5 µg of total RNA (in a final volume of 20 µl) and was carried out for 10 min at 25 °C followed by 120 min at 37 °C employing reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The reaction was stopped at 85 °C for 5 min, using a thermal cycler (Eppendorf, Westbury, NY, USA). The primers employed for qRT-PCR analyses of target gene expression are provided in our previous publications [6, 7]. 18S RNA was employed as the reference housekeeping gene used to normalize mRNA expression.

qRT-PCR was performed in a total volume of 20 µl, including 40 ng of cDNA template, forward and reverse primers (100 nM each), and PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) using a LightCycler 480 instrument (Roche, Branchburg, NJ, USA). After initial denaturation and enzyme activation (95 °C for 10 min), 40 cycles (94 °C for 10 sec, 55 °C for 30 sec, 72 °C for 30 sec) were performed for the annealing/extension steps, and fluorescence was measured. A dissociation curve program was performed after each cycle. Expression levels of target genes were calculated based on the efficiency of each reaction and the crossing point deviation of each sample versus a control and are expressed as fold differences in comparison with the reference gene.

2.4. UPLC/MS/MS analysis of lipids

All measurements were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA, USA). The system was controlled by Mass Lynx Software version 4.1 (Waters, Milford, MA, USA). Free fatty acids, ceramides, endocannabinoids and phosphatidylcholine were extracted from HSCs using chloroform:methanol. Briefly, 3 ml of pre-cooled 2:1 chloroform:methanol (v/v) and 20 µl of internal standards cocktail in methanol containing 1 mM deuterated palmitic acid-d31 (C/D/N Isotopes Inc., Quebec, Canada), 2 µM C12 ceramide and 2 µM C25 ceramide (Avanti Polar Lipids, Inc., Alabaster, AL, USA), 1 µM arachidonylethanolamide-d8 and 5 µM 2-arachidonoylglycerol-d8 (Cayman Chemical Co., Ann Arbor, MI, USA) and 1 mM of C17/C17 phosphatidylcholine (Avanti Polar Lipids, Inc., Alabaster, AL, USA) were added to 50 µL of cell suspension containing 2×10^5 cells in a clean glass tube. This was mixed well in a vortex mixer. One-half milliliter of water was added to the mixture to allow for phase separation and the mixture was vortexed well again and centrifuged at 3,000 g for 10 min to separate phases. The lower chloroform phase was transferred to another clean glass tube using a Pasteur pipette. Two ml of precooled chloroform was added to the remaining upper aqueous phase and this was mixed well and again centrifuged at 3,000 g for 10 min to separate phases. The chloroform phases were pooled and evaporated to dryness under N₂. The lipid extract was reconstituted in 50 µl acetonitrile and transferred to an autosampler vial (Waters, Milford, MA, USA) for processing by liquid chromatography mass spectrometry. Retinoids (retinol + retinyl esters) were extracted from HSCs with n-hexane. Briefly, 50 µL of cell suspension containing 2×10^5 cells in a clean glass tube was mixed with 50 µL of ethanol containing 1 µM retinyl acetate (Millipore Sigma, Milwaukee, WI, USA). This was mixed well in a

vortex mixer and 0.4 mL of 1:1 water:EtOH (v:v) was added to the sample followed by additional mixing in a vortex mixer. Two ml of pre-cooled n-hexane was added and the mixture was vortexed again and centrifuged at 3,000 g for 5 min to separate phases. The upper organic phase was transferred to a second clean glass tube using a Pasteur pipette, then evaporated under nitrogen. The resulting lipid film was resuspended in 100 μ L of acetonitrile and then transferred to another glass tube followed by evaporation under nitrogen. The dried lipid was reconstituted in 50 μ L of methanol and transferred to LC autosampler vials for injection. Samples in autosampler vials were maintained at 4 °C in the autosampler until 5 μ L was injected onto a Waters ACQUITY UPLC column.

A BEH C18 column (2.1 $\times$ 100 mm, 1.7 μ m particle size, Waters), preceded by a 2.1 $\times$ 5 mm guard column using the same packing material, was employed for free fatty acid. A BEH Phenyl column (3 mm inner diameter $\times$ 100 mm with 1.7 μ m particles), preceded by a 2.1 $\times$ 5 mm guard column containing the same packing, was used for ceramide, phosphatidylcholine, and retinoids. An HSS C18 column (2.1 mm inner diameter $\times$ 100 mm with 1.8 μ m particles), preceded by a 2.1 $\times$ 5 mm guard column containing the same packing, was used for endocannabinoid. All columns were maintained at 40 °C. All flow rates were 300 μ L/min in a binary gradient mode.

For *fatty acid* analyses, the samples were diluted 20-times with methanol before injection. The UPLC mobile phases were initiated using 15% solvent A [100% H₂O] and 85% solvent B [100% methanol]. The concentration of solvent B was increased linearly to 100% over 8 min and maintained at 100% until 10 min. The gradient was then re-equilibrated to 15% solvent A and 85% solvent B over 2 min before the next sample was injected. Negative ESI-MS using the selected ion recording (SIR) mode was performed, employing the following parameters: capillary voltage -3.8 kV; source temperature 150 °C; desolvation temperature 500 °C; and desolvation gas flow 1000 l/hr. Optimized cone voltages ranged from 36 to 52 V and the following mass (m/z) were employed: myristic acid 227.2, myristoleic acid 225.2, palmitic acid 255.2, palmitoleic acid 253.2, stearic acid 283.3, oleic acid 281.3, linoleic acid 279.2, linolenic acid 277.2, eicosanoic acid 311.2, eicosenoic acid 309.3, eicosadienoic acid 307.3, arachidonic acid 303.2, eicosapentaenoic acid 301.2, behenic acid 339.3, docosenoic acid 337.3, docosahexaenoic 327.2, tetracosanoic acid 367.4, tetracosenoic acid 365.3 and palmitic acid-d31 286.5.

To assess the *fatty acyl composition of triglycerides*, HSCs cultured for different times were washed with PBS three times, followed by addition of 2 ml of 3:2 n-hexane:isopropanol (v/v). Cells were left for 2 hours for lipid extraction and subsequently n-hexane:isopropanol extracts were collected into glass tubes. An additional 1 ml of 3:2 n-hexane:isopropanol (v/v) was added to each well and left for extraction for 30 min. n-Hexane:isopropanol extracts were collected and combined with the first extraction. The samples were dried under N₂, 0.5 ml of hexane was added to each sample and the samples were vigorously vortexed. An aliquot of the hexane extract from each sample was dried under N₂ and used for total TG concentration assessment using Infinity™ Triglyceride kit (Thermo Scientific, Middletown, VA, USA). Lipid extracts were spotted on a MiliporeSigma TLC Silica gel 60 plastic sheet (20 $\times$ 20 cm). Lipids were separated by thin-layer chromatography employing a mixture of 70:30:1 hexane:diethyl ether:acetic acid (v/v) as a mobile phase followed by lipid

visualization in an iodine chamber. The separated TGs were scraped and transferred to a glass tube with 1 ml of ethanolic KOH containing 20 μ l of 100 μ M deuterated palmitic acid-d31 (C/D/N Isotopes Inc., Quebec, Canada) as an internal standard. After incubation at 60 °C for 1 h, 0.5 ml of H₂O and 0.4 ml of hexane were added and the phases were separated by centrifugation. The nonsaponifiable lipids in the upper hexane phase were removed, and the pH of the lower phase was titrated with 1 N HCl to pH 2.5. The unesterified fatty acids were then extracted into n-hexane and analyzed by LC/MS as described above. All analyses were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters).

For *endocannabinoid* analyses, the UPLC flow rate was isocratic composed of 17% solvent A (H₂O containing 0.1% formic acid) and 83% solvent B (acetonitrile containing 0.1% formic acid). The UPLC running time was 4 minutes and endocannabinoids of interest eluted between 2 and 3.5 min. Positive ESI-MS/MS was performed employing the following parameters: capillary voltage, 3.8 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 l/hr; and collision gas flow, 0.18 ml/min. The optimized cone voltage was 22 V, collision energy for multiple reaction monitoring mode was 14 eV. The following transitions (m/z) were employed: AEA 348.3→62.0, AEA-d8 356.3→63.0, 2-AG 379.3→287.3, 2-AG-d8 387.3→294.2.

For *ceramide* analyses, initial flow conditions were 20% solvent A (H₂O, containing 0.2% formic acid and 1 mM ammonium formate) and 80% solvent B (methanol, containing 0.2% formic acid and 1 mM ammonium formate). Solvent B was increased linearly to 95% over a 2 min period and to 98% in the subsequent 6 min. This was followed by a reduction of solvent B to 80% starting at 8.2 min and continuing through 9 min. Ceramides of interest eluted between 4.0 and 7.5 min. Positive ESI-MS/MS was performed using the following parameters: capillary voltage, 3.8 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 l/hr; and collision gas flow, 0.18 ml/min. The optimized cone voltage was 22 V, collision energy for MRM mode was 26 eV. The following transitions (m/z) were employed: C12 ceramide 482.55→264.2, C14 ceramide 510.45→264.2, C16 ceramide 538.48→264.2, C18:1 ceramide 564.5→264.2, C18 ceramide 566.51→264.2, C20:1 ceramide 592.6→264.2, C20 ceramide 594.54→264.2, C22:1 ceramide 620.7→264.2, C22 ceramide 622.64→264.2, C24:1 ceramide 648.59→264.2, C24 ceramide 650.61→264.2, C25 ceramide 664.68→264.2, C26 ceramide 678.8→264.2.

For *phosphatidylcholine* analysis, the sample was diluted 100-times with methanol before injection. The UPLC flow rate was 500 μ l/min in a binary gradient mode with the following mobile phase gradient: initiated with 8% phase A (water containing 0.2% formic acid and 1 mM ammonium formate) and 92% mobile phase B (methanol containing 0.2% formic acid and 1 mM ammonium formate). Gradient of methanol was increased linearly to 100% over 6 min. The column was washed with 100% methanol for 2 minutes and the recondition with the initial solvent for half a minute before the next injection. The phosphatidylcholines of interest eluted between 1.5 and 5 min. Positive ESI-MS/MS with multiple reaction monitoring (MRM) mode was performed using the following parameters: capillary voltage, 3.85 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow,

1000 L/hr; and collision gas flow, 0.18 mL/min. The optimized cone voltage was 42 V, collision energy for multiple reactions monitoring mode (MRM) was 30 eV. For MRM analysis, m/z of $[M+H]^+$ was used for each parent ion, and m/z of 184.2 was used for all the daughter ions.

For **retinoid** (retinol + retinyl esters) analyses, the flow rate was 500 μ L/min in a binary gradient mode with the following mobile phase gradient: initiated with 5% phase A (water containing 0.2% formic acid and 1 mM ammonium formate) and 95% mobile phase B (methanol containing 0.2% formic acid and 1 mM ammonium formate). The gradient of methanol was increased linearly to 97.5% over 5 min. The column was then washed with 99% methanol for 1 minute and the next sample injected. Positive ESI-MS mass spectrometry with selected ion recording (SIR) mode was performed using the following parameters: capillary voltage -4.0 kV, source temperature 150 $^{\circ}$ C, desolvation temperature 500 $^{\circ}$ C, desolvation gas flow 1000 L/hr. Retinol and retinyl esters were detected using m/z 269.25 (retinol dehydrate $[M+H]^+$) and identified by comparing the retention times of experimental compounds with those of authentic standards.

The concentrations of individual lipid species measured were normalized to cellular DNA concentration. The data presented for TG acyl groups, are expressed as a percentage of each fatty acid.

2.5. Statistical analysis

All data are presented as means $\pm$ S.D. Student's t -test was used to analyze differences between lipid concentrations determined for freshly isolated HSCs (day 0 values) and for HSCs at different times of culture (the data obtained for day 0.5, 3 and 7). Statistical comparisons of mRNA fold changes at different time points were first analyzed by a one-way ANOVA followed by multiple comparisons employing Tukey's HSD post hoc test. P values less than 0.05 were considered to be statistically significant.

3. RESULTS

Culturing primary mouse HSCs cells on a plastic surface resulted in their activation. This was accompanied by pronounced changes in cell morphology (Fig. 1A). During the transition from a quiescent state (freshly isolated cells), HSCs progressively lost their characteristic lipid droplets acquiring a myofibroblast-like phenotype. HSCs activation was further confirmed by the presence of specific activation markers [8]. A significant upregulation of mRNA expression of *Acta2*, a gene encoding α -smooth muscle actin, was detected as early as in overnight HSC cultures followed by its 1000- and 2000-fold increase at day 3 and day 7, respectively (Fig. 1B). Transcript levels for another activation marker *Col1a1*, a gene encoding type I collagen involved in extracellular matrix formation, were 20-fold elevated in 3-day cultures and by more than 50-fold in 7-day HSCs cultures (Fig 1B).

Elevated mRNA expression of extracellular matrix proteins during activation of primary HSCs on plastic was accompanied with a decline in total retinyl ester (RE) levels (Fig. 1C). This was also accompanied by a 10-fold downregulation of mRNA expression of *Lrat* (Fig. 1D), a gene encoding the sole protein responsible for hepatic RE synthesis and HSC lipid

droplet formation [9]. A rapid decline in RE concentrations that were reduced by over 60% in the first 12 hrs of culture and a more than 90% drop in REs by day 3 of culture was accompanied by an upregulation in transcription of retinoic acid responsive genes. Message levels for *Rarβ*, a gene encoding β isoform of retinoic acid receptor that enables retinoic acid transcriptional activity, and *Cyp26b1*, a gene encoding cytochrome P450 isoform responsible for retinoic acid catabolic oxidation in HSC, were significantly elevated at all of the times of HSC culture (Fig. 1D). The differences were statistically significant, starting in overnight cultures, and became even more pronounced by day 7 of HSC culture (Fig. 1D). These transcriptional changes are known to be a result of enhanced formation of retinoic acid arising from the enzymatic oxidation of retinol being produced from REs present in HSC lipid droplet [4].

HSC activation was associated with a decline in concentrations of cellular TGs (Fig. 2A), which represent the second most abundant lipid group within HSC lipid droplets. Significant changes in TG concentrations started to be detected at day 3 of HSC culture, declining to a level of only 15% of the day 0 level by day 7 of culture (Fig. 2A). During HSC activation, we did not observe marked changes in the fatty acyl composition of TGs (Fig. 2B and 2C). Saturated (C12:0-C20:0) acyl groups account for almost 95% of all acyl groups identified in HSC TGs, with palmitate and stearate being the most abundant. This did not change substantially throughout the period of cell culture (Fig. 2B and 2D). During HSC activation the percentage of monounsaturated (C18:1-C20:1) acyl groups remained at a level of 2.5-3.5%, while the percentage of polyunsaturated acyls (C20:2-C22:6) was significantly increased from 0.16% in quiescent HSCs (freshly isolated) to 1.26% in activated HSCs (day 7) (Fig. 2B and 2D).

Upon overnight culture, mRNA expression of proteins involved in the lipid droplet-associated hydrolysis of triglycerides, the predominant non-retinoid lipid species present within HSC lipid droplets, either increased (*Atgl*, *Tgh1*) or remained constant (*Hsl*) (Fig. 2E). mRNA expression of *Atgl* remained elevated during all of the times of HSCs culture, whereas *Tgh1* expression declined on day 3 of culture and was lower than in quiescent freshly isolated HSC on day 7 of culture. mRNA levels of *Dgat1* and *Dgat2*, genes encoding the two distinct acyltransferases that catalyze the last step of triglyceride synthesis, were respectively 6- and 1.5 fold higher in overnight cultures compared to freshly isolated HSC (Fig. 2F). *Dgat1* mRNA expression remained elevated during all 7 days of culture, whereas *Dgat2* message levels declined on day 3 and 7 of culture (Fig. 2F).

mRNA expression of *Lc3b*, a gene encoding a protein involved in autophagosome formation implicated in lysosome-mediated lipid droplet breakdown [10], was significantly upregulated in overnight cultures compared to freshly isolated HSCs (Fig. 2F). However, *Lc3b* transcript levels declined at day 3 and day 7 of culture returning to the level of quiescent HSCs (Fig. 2F).

The significantly higher levels of *Atgl* and *Tgh1* mRNA expression in overnight cultures suggest a role in the hydrolysis of neutral lipid during initiation of HSC activation, resulting in free fatty acid mobilization from the lipid droplets. To understand this better, a targeted lipidomic approach was employed to analyze HSC free fatty acid concentrations.

UPLC/MS/MS analysis revealed an increase in total intracellular free fatty acid (FFA) levels in cultured HSCs following their activation on plastic. The maximal total free fatty acid concentration was observed in overnight cultures followed by a subsequent decline at days 3 and 7 (Fig. 3A). The significant elevation of total free fatty acid concentrations observed in overnight HSC cultures, was predominantly due to higher levels of C16 and C18 fatty acid species. The highest concentrations were determined for palmitate (C16:0), followed by oleate (C18:1), palmitoleate (C16:1), linoleate (C18:2) and stearate (C18:0). As the culture period increased, the absolute concentrations of these fatty acid species declined (Fig. 3B and 3C) resulting in the observed decline of total free fatty acid concentration compared to quiescent freshly isolated HSCs (Fig. 3A). However, the concentrations of long chain free fatty acids, including eicosanoic (C20:0), docosanoic (C22:0), tetracosanoic (C24:0), hexacosanoic (C26:0), eicosapentanoic (C20:5), arachidonic (C20:4), and docosahexaenoic (C22:6) acids, remained relatively high or even became elevated at day 3 of culture compared to levels of these fatty acids in freshly isolated HSCs (Fig. 3B and 3C). Most strikingly, the level of arachidonic (AA) and docosahexaenoic (DHA) acids remained significantly elevated even at day 7 of HSCs culture. The fraction of these two polyunsaturated fatty acids (PUFA) in the total fatty acid pool at day 7 reached 20% and 2% respectively compared to 0.23% and 0.22% in freshly isolated HSCs. The relative fraction of palmitic acid remained constant (at about 40%) and the fraction of oleic acid declined from 27% to 11% following activation (Fig. 3A–3C). The increase in long chain highly unsaturated fatty acid concentrations during HSCs activation was accompanied by an amplification of mRNA levels of enzymes involved in fatty acid desaturation (*Scd1* and *Scd2*) and elongation (*Elovl5* and *Elovl6*) (Fig. 3D). Taken together, these findings suggest that culture activated primary mouse HSCs show increased formation of long chain polyunsaturated fatty acids during *in vitro* activation.

Since highly unsaturated long chain fatty acids are known to function as structural components of bioactive lipids that mediate many essential cellular functions, specifically those related to stress reactions like the ones reported to occur during HSC activation [11, 12], we narrowed our interest to a number of classes of lipid mediators containing PUFAs, specifically focusing on endocannabinoids and the related N-acyl ethanolamides (NAEs), as well as ceramides. Because phosphatidylcholine (PC) is a common precursor in the biosynthesis of these lipid mediators, PC concentrations were investigated in HSC cultures during plastic activation. HSC PC levels increased upon overnight cultures compared to freshly isolated HSCs (Fig. 4A). This elevation was mostly due to elevated levels of PC species 34:2 (containing predominantly fatty acid moieties C16:0-C18:2, C16:1-C18:1), followed by 34:1 (C16-C18:1, C16:1-C18), 36:4 (C18:2-C18:2, C18:1-C18:3, C16-C20:4) and 38:6 (C18:2-C20:4, C18:1-C20:5, C16-C22:6). This was followed by a significant decline in total PC concentrations by day 7 (Fig. 4A–4C). The concentration of 34:2 PC species diminished, while the level of 34:1 PC remained constant. Concentrations of 36:4 and 38:4 PC species remained relatively high, placing these PC species, which are enriched with highly unsaturated fatty acid moieties, among the predominant phosphatidylcholines present in HSC cultures at day 7. Moreover, analysis of concentrations of individual PC species revealed that the levels of 36:6 PC (C18:3-C18:3, C16:1-C20:5, C14-C22:6), 38:4 PC (C18-C20:4, C18:3-C20:1) and C44:10 PC (C22:5-C22:5) were elevated the most at day

7 of culture compared to freshly isolated HSCs (Fig. 4B and 4C). Taken together, these observations imply an enrichment of PCs with polyunsaturated fatty acid moieties along with a progression of HSC *in vitro* activation.

We next investigated possible concentration changes in the lipids that are either synthesized from PC or contain a PUFA moiety. The analysis of endocannabinoid concentrations revealed that 2-AG was the most abundant of the two canonical endocannabinoid species identified in HSC cultures. An increase in 2-AG concentrations was observed in overnight and 3-day HSC cultures (Fig. 5A–5C). This was paralleled by an elevation of *Dagla* mRNA (Fig. 5D), which encodes the Sn1-specific diacylglycerol lipase that is capable for generating 2-AG from diacylglycerol. HSC levels for the other canonical endocannabinoid, N-arachidonylethanolamide (AEA) were several orders of magnitude lower than those of 2-AG but were significantly elevated in overnight HSC cultures, declining at days 3 and 7 of culture. Surprisingly, the NAE, N-palmitoylethanolamide (PEA) was identified as the second most abundant endocannabinoid-like species in HSCs during activation in culture. The concentrations of this potent NAE increased in overnight HSC cultures and remained elevated throughout the 7-day culture period. The elevated levels of PEA at days 3 and 7 of HSC cultures were associated with an elevation in mRNA levels encoding *Nape-pld*, an enzyme that hydrolyzes N-acyl-phosphatidylethanolamines (NAPEs) to produce NAEs like PEA. This was accompanied by diminished levels of *Faah* transcripts encoding an enzyme that degrades bioactive fatty acid amides and thus terminates the signaling functions of these molecules (Fig. 5D).

An increase in total ceramide levels was identified in HSCs undergoing activation, reaching a maximum at day 3 compared to freshly isolated primary cells (Fig. 6A–C). During activation, HSCs were enriched with N-palmitoyl ceramide (the C16:0 ceramide species), followed by N-tetracosenoyl ceramide (C24:1), N-tetracosanoyl ceramide (C24:0) and N-docosanoyl ceramide (C22:0). The concentration of these ceramide species as well as the concentration of all 12 ceramides analyzed trended towards elevated ceramide concentrations peaking at day 3 of HSC culture (Fig. 6B). These changes were preceded by increased mRNA expression of ceramide synthesizing enzymes, including *CerS2* (which generates C22–C24-ceramides), *CerS5* (which generates C16 ceramides) and *Smpd1* (which generates ceramides from sphingomyelins) (Fig. 5D).

4. DISCUSSION

HSC activation is known to play a crucial role in promoting the pathogenesis of liver disease, including hepatic inflammation, fibrosis, cirrhosis and hepatocellular carcinogenesis. HSC *in vivo* activation is considered to be the gold standard for study of HSC biology, however *in vitro* culture of primary stellate cells isolated from the liver is widely employed as an experimental approach to gain understanding HSC activation pathways. The primary cellular event accompanying HSC activation and preceding all major visible changes associated with activated HSCs and is a loss of their characteristic retinoid-containing lipid droplets (Fig. 1A). Numerous efforts aimed at explaining the physiological relevance of the phenomenon of lipid droplets loss and the fate of the lipids released during HSC activation have been reported in the literature [4, 13, 14]. The importance of lipid

droplet-derived retinoids in maintaining liver-specific responses to hepatic disease development have been extensively studied by many investigators [6, 15–18]. However, less attention has been focused on the roles of non-retinoid lipid species, which account for more than 60% of the total lipids found in HSC lipid droplets [2]. In the current study, using cultures of mouse HSCs and state-of-the-art UPLC/MS/MS methodology we expand understanding of other important signaling non-retinoid lipids during HSC activation.

Our data establish that primary mouse HSCs undergoing activation during culture on a plastic surface generate highly potent bioactive non-retinoid lipid species. These newly synthesized lipids, including long chain polyunsaturated fatty acids (PUFAs), endocannabinoids and related NAEs, and ceramides, are formed during HSCs lipid droplet breakdown and remodeling, likely contribute to the transition of HSCs from quiescence to the activated state or, once released, contribute to signaling to surrounding cells.

We show an increase in total free fatty acid concentrations during the early stage of HSC activation and a subsequent decline in later stages. UPLC/MS/MS analysis of the concentrations for 20 individual C12-C22 fatty acid species (Fig. 3) confirmed that the most abundant free fatty acids, comprising palmitic, oleic, palmitoleic and stearic acids, reach peak concentrations early in the activation process, as the HSCs begin to express activation markers and to lose their ability to maintain retinyl ester stores. This rise in levels of free fatty acids may arise from lipid droplet-associated triglycerides and retinyl esters which undergo extensive hydrolysis in activated HSCs, and is accompanied by an elevation in mRNA expression of *Atgl* which plays a role in the triglyceride hydrolysis during HSCs activation [19]. Taken together the changes in fatty acid concentrations and gene expression likely reflect an increase in lipid droplet lipolysis and lipid turnover that is initiated early as the HSCs transition from the quiescent to the activated state.

The physiological relevance of fatty acid mobilization due to the hydrolysis of lipid droplets and its role in HSCs activation are not well understood. It has been proposed in the literature that free fatty acids being liberated from lipid droplets undergo mitochondrial β -oxidation thus providing energy that is essential to support stellate cell activation [20]. Indeed, uncoupling of mitochondrial respiration has been shown to inhibit *in vitro* activation of mouse and human HSCs, leading to diminished α -SMA expression and HSCs proliferation [21]. Both lipid droplet-associated and lysosomal pathways of lipolysis, involving neutral and acidic lipases respectively, have been proposed to be implicated in an acquisition of FFA by HSCs during activation [10, 14, 19, 22]. ATGL is thought to be the major, but not the sole hydrolase among the neutral lipases, involved in hydrolysis of lipid droplet-associated triglycerides (TGs) in HSCs [19, 22]. However, the studies employing HSCs with either *Atgl* gene knockout or chemical inhibition of ATGL activity revealed only higher accumulation of lipid droplet-associated TGs, but no differences in the rate of TGs hydrolysis during HSCs activation [19, 22]. At the same time, autophagy, a process involving a formation of double-membrane autophagosome with its subsequent fusion with a lysosome to form an autolysosome in which the autophagosome cargo is mixed with and degraded by the lysosomal enzymes for release back into the cytosol [23], has been shown to play a critical role in HSCs activation [10, 14]. Blocking autophagosome formation in HSCs through either utilization of pharmacologic inhibitors (3-methyladenine or chloroquine) or specific *Atg5* or

Atg7 knockdown resulted in inhibition of lipid droplet degradation and significantly reduced the expression of messenger RNAs encoding activation markers and corresponding proteins [14]. Likewise, lalistat-mediated inhibition of lysosomal acid lipase, an enzyme that functions downstream the autophagosome formation, attenuated murine HSCs activation *in vitro* [24]. Conversely, enhancing autophagy flux as well as triggering a production of light chain 3-II protein and autophagosomes via treatment of cells with 200 μ M palmitic acid has been shown to elicit activation of primary rat HSCs and immortalized stellate cell lines, including human LX2, human immortalized HSCs, and rat BSC-C10 cells [25]. Consistent with these reports, our data indicating elevation in mRNA levels for neutral lipases and *Lc3b* (Fig. 2E and 2E) imply that both lipid droplet-associated and lysosomal pathways of lipolysis may account for the observed increase of FFA during initial periods of HSCs activation. The need for FFAs as an energy source during initiation of HSCs activation may be a valid explanation for the observed elevation of FFA during the early periods of HSCs culture. However, our data discussed below suggest that some fatty acids derived from HSC lipid droplet remodeling may also be implicated in pathways that are distinct from ones utilized to provide energy.

At later stages of HSC activation, there is a decline in the concentrations of the very abundant free fatty acid species that are likely used as energy substrates. However, while we observed a decline in concentrations of both total as well as the major free fatty acid species present in HSCs during activation, we observed an elevation in the levels of specific C20-C22 species, especially arachidonic and docosahexaenoic acids. Furthermore, the relative abundance of these PUFA species in the general intracellular free fatty acid pool increased dramatically, causing these fatty acids to become highly abundant in activated HSCs (Fig. 3B and 3C). An upregulation of transcription of genes encoding fatty acid elongases (*Elovl5* and *Elovl6*) and desaturates (*Scd1* and *Scd2*) in activating HSCs (Fig. 3D) implies a shift in metabolism towards the synthesis and accumulation of long chain polyunsaturated fatty acids during activation. In accordance with our data, *Scd1* and *Scd2* have been identified among upregulated genes in published studies using microarray analysis of the analogously culture-activated primary mouse HSCs [26, 27]. Furthermore, the relevance of *Scd1* upregulation identified in the *in vitro* system was confirmed by elevation of the gene expression in HSCs isolated from fibrotic livers of either bile duct-ligated or CCl₄-treated mice [26]. Taken together these published observations and our data indicating PUFAs elevation support the idea that activated HSCs are able to maintain fatty acid processing, including preferential elongation and desaturation.

The phenomenon of PUFAs accumulation has been reported in the literature for murine HSCs during their activation *in vitro* [13, 22, 28]. However, in these studies PUFAs containing more than 20 carbons and 3 double bonds were identified to be preferentially incorporated into triglycerides (PUFA-TGs) [13, 28]. Employing HPLC-MS analysis of neutral lipid and phospholipid fractions derived from on silica-G column separation of HSCs lipid extracts and assessing the percentage of TG ion fragments in the *m/z* 859–1003 region in these lipid fractions allowed the authors to conclude that in activated HSCs the total amount of PUFAs incorporated in the neutral lipids becomes similar to the total amount of PUFAs in the phospholipid fraction [28]. It was proposed that in the activated HSCs the majority of PUFA-enriched TGs is associated with newly synthesized lipid droplets. These

lipid droplets were proposed to be distinct from “pre-existing” lipid droplets of quiescent HSCs, being much smaller in size, devoid of RE and displaying a high TG turnover, mediated by ATGL and DGAT1 [22]. Despite providing greater knowledge of the turnover of lipid droplet-associated TGs during HSCs activation *in vitro*, these studies provide limited insight into the physiological role and function that TG-incorporated PUFAs might play in hepatic stellate cell biology. It has been proposed that PUFAs associated with the neutral lipids play a role in replenishing the phosphatidylcholine pool in activated HSCs in order to sustain eicosanoid production [28]. In our study, employing UPLC/MS/MS analysis of fatty acids derived from TGs after their saponification, we did not detect marked changes in the fatty acyl composition of TGs during HSC activation. While we did observe an increase in percentage of TG-associated PUFAs, this elevation did not exceed more than 2% of the total TG acyl groups pool (Fig. 2B). At the same time, our study establishes that the fraction of unesterified arachidonic (AA) and docosahexaenoic (DHA) acids in the total free fatty acid pool at day 7 of HSCs activation reached 20% and 2% respectively compared to 0.23% and 0.22% in freshly isolated HSCs (Fig. 3). Our data demonstrate that the periods of HSCs activation that are characterized with elevated levels of PUFAs, including AA and DHA (Fig. 3.), are followed by an enrichment of PCs with polyunsaturated fatty acid moieties (Fig. 4). UPLC/MS/MS analysis revealed a significant raise in the relative concentrations of phosphatidylcholine species 36:4, 36:6 and 38:4, all of which contain C20:4 and C22:6 fatty acids as structural components (Fig. 4). Overall, these data support the notion that there is increased formation and redistribution of long chain and PUFA species in activated HSCs. In addition, our data suggest that this process is an important intermediate step in the pathways aimed at providing PUFA-containing bioactive lipid mediators during HSCs activation.

In order to monitor concentration changes of lipid mediators that may comprise fatty acids being redistributed and processed during HSCs activation, intracellular concentrations of endocannabinoids and related NAEs, and ceramides were analyzed. Among the 7 endocannabinoid and NAE species analyzed, 2-arachidonoyl glycerol (2-AG) was the most abundant species. The concentration of the other canonical endocannabinoid, AEA, was lower by three orders of magnitude compared to 2-AG levels (Fig. 5). However, both 2-AG and AEA displayed increased levels during the early stage of HSCs activation along with upregulated mRNA expression of the genes encoding endocannabinoid metabolizing enzymes (Fig. 5). An increase in 2-AG concentrations observed in overnight and 3-day HSC cultures was paralleled by an elevation of *Dagla* mRNA (Fig. 5D), which encodes the Sn1-specific diacylglycerol lipase that is capable for generating 2-AG from diacylglycerol. However, the expression of *Nape-pld*, a gene encoding a protein that is considered to be a major contributor in NAE formation, was increased after 3 days of culture, while the cellular NAE concentration was already increased after 0.5 days of culture. This inconsistency between *Nape-pld* mRNA expression and NAE accumulation suggests that other pathways of endocannabinoid production in activated stellate cells may exist, given reports suggesting that NAEs can be produced through multi-step pathways bypassing NAPE-PLD [29].

A substantial literature has implicated the two canonical endocannabinoids as being crucial regulators of hepatic fibrogenesis, a condition representing *in vivo* activation of HSCs [12]. 2-AG has been proposed to act as a fibrogenic mediator, because its hepatic level is preferentially increased during either ethanol feeding [30] or upon CCl₄ treatment [31] of

mice. Likewise the endocannabinoid receptor CB₂, which binds 2-AG with high affinity mediating 2-AG signaling, is normally undetectable in the liver, but is highly expressed in cirrhotic human liver and in non-parenchymal liver cells from fibrotic mouse liver [32]. In agreement with our data, picomolar concentrations of 2-AG have been reported for HSCs isolated from the livers of ethanol-fed mice [30]. Moreover, this HSC-derived 2-AG, in addition to mediating HSCs activation, has been shown to act as a paracrine activator of lipogenic program in adjacent hepatocytes [30], implying its contribution to the development of the hepatic disease. Taken together, our data and the published literature underscore the formation of the arachidonate-based bioactive lipid in activated HSCs within picomolar concentration range. Importantly, the concentrations of the newly generated endocannabinoids may affect the responses of activated HSCs to these bioactive lipids. A number of *in vitro* studies indicate that culture-activated murine HSCs are highly susceptible to endocannabinoid-induced cell death when the cells are treated with micromolar concentrations of either 2-AG or AEA [31, 33, 34].

An unexpected finding from our analysis of NAEs was the identification of PEA as the second most abundant endocannabinoid/NAE species present in activating HSCs. The finding that *in vitro* activated HSCs become enriched with PEA is consistent with a report indicating significantly higher levels of this ethanolamide in the circulation of patients with cirrhosis [35]. Interestingly, PEA, unlike the canonical endocannabinoids AEA and 2-AG, has been identified as a ligand for PPAR α , one of the master regulators of hepatic lipid metabolism [36]. PPAR α has been implicated in mediating antiinflammatory actions of PEA; however, the underlying mechanisms and the physiological relevance of this NAE in activated HSCs remains to be elucidated. Collectively, our data provide quantitative evidence of substantial formation of selective endocannabinoids and related NAEs during HSCs activation.

Ceramide (N-acylsphingosine) metabolism is becoming a focus for the development of anti-fibrogenesis strategies [37–39]. Our data establish that ceramide accumulation and the upregulation of genes involved in ceramide synthesis as events that are associated with HSC activation (Fig. 6). This is consistent with reports implicating sphingolipids in HSCs activation [37, 38]. It has been shown that selective stimulation of ceramide synthesizing acidic sphingomyelinase (ASMase), but not neutral sphingomyelinase, is taking place during the transdifferentiation/activation of primary mouse HSCs into myofibroblast-like cells. These studies also demonstrated that ASMase inhibition or genetic down-regulation by small interfering RNA could prevent the activation and proliferation of mouse and human HSCs [37, 38]. In our study, we detected an upregulation of *Smpd1* expression, a gene encoding ASMase that generates ceramides from sphingomyelins, in cultured primary mouse HSCs during activation. Additionally, we have identified a significant upregulation in mRNA expression of the ceramide synthases *CerS2* and *CerS5*, which are involved in generating C22-C24- and C16-ceramides respectively. These transcriptional changes were associated with an accumulation of predominant N-palmitoyl ceramide (the C16:0 ceramide), but also with less abundant long chain fatty acid-containing ceramides, including N-tetracosenoyl ceramide (C24:1), N-tetracosenoyl ceramide (C24:0) and N-docosanoyl ceramide (C22:0). The preferential accumulation of C22-C24 acyl-containing ceramides

allows for speculating that these species may comprise long chain fatty acids selectively generated during HSCs activation.

Overall, our data provide experimental evidence of preferential accumulation of long chain and polyunsaturated fatty acids as well as the bioactive lipids comprising these species in HSCs during their *in vitro* transition from the quiescent to activated state. However, further studies will be required to delineate specific pathways leading to the formation of these fatty acid species, endocannabinoids and ceramides and their role in HSCs activation *in vivo*.

5. CONCLUSIONS

Taken together, our data indicate that lipid droplet breakdown associated with HSC activation is not simply a passive lipolysis aimed at riding the cell of inert lipid content. Rather, this is an active process underlying the redistribution or/and the formation of low abundance, highly bioactive lipid species that serve as signaling lipid mediators within the liver. Our findings employing a model of mouse HSCs activation in culture coupled with UPLC/MS/MS analysis of cellular lipids expand knowledge of HSC biology. However, a question remains as to whether the changes we observed in the lipid mediators enable HSCs adaptation during stress-induced activation or rather should be considered simply as a step needed towards delivering lipid mediators to serve in an autocrine or paracrine manner, thus contributing to the development of cell- and liver-specific responses in hepatic disease.

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ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
Acta2	actin, aortic smooth muscle
AEA	N-arachydonylethanolamide
ASMase	acidic sphingomyelinase
Atgl	adipose triglyceride lipase
CerS2	ceramide synthase 2
CerS5	ceramide synthase 5
Coll1a1	collagen alpha-1(I) chain
Cyp26b1	cytochrome P450 26B1
Dagla	diacylglycerol lipase alpha
Dgat1	diacylglycerol O-acyltransferase 1

Dgat2	diacylglycerol O-acyltransferase 2
DHA	docosahexaenoic acid
Elov15	elongation of very long chain fatty acids protein 5
Elov16	elongation of very long chain fatty acids protein 6
Faah	fatty acid amide hydrolase 1
FFA	free fatty acid
HSC	hepatic stellate cell
Hsl	hormone-sensitive lipase
Lc3b	microtubule-associated protein light chain 3B
Lrat	Lecithin retinol acyltransferase
NAE	N-acylethanolamide
NAPE	N-acyl-phosphatidylethanolamine
Nape-pld	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D
PC	phosphatidylcholine
PEA	N-palmitoylethanolamide
PUFA	polyunsaturated fatty acids
Rarβ	retinoic acid receptor beta
RE	retinyl ester
Scd1	acyl-CoA desaturase 1
Scd2	acyl-CoA desaturase 2
Smpd1	sphingomyelin phosphodiesterase
TG	triglyceride
TGFβ1	transforming growth factor beta-1
Tgh1	triacylglycerol hydrolase

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HIGHLIGHTS

- Culture-activated primary mouse HSCs are enriched with unesterified and phosphatidylcholine-incorporated arachidonic and docosahexaenoic acids.
- 2-Arachidonoylglycerol (2-AG) and N-arachidonylethanolamide levels are increased during the early stage of HSCs activation.
- 2-AG is the predominant endocannabinoid and N-palmitoylethanolamide is the second most abundant endocannabinoid-like species in HSCs during culture activation.
- During activation, HSC cultures are enriched with N-palmitoyl (C16:0), N-tetracosenoyl (C24:1), N-tetracosanoyl (C24:0) and N-docosanoyl (C22:0) ceramides and this is accompanied by an upregulation of *CerS2*, *CerS5* and *Smpd1* transcription.

Figure 1. Hepatic stellate cell activation markers during *in vitro* culture.

Panel A – A representative microphotograph of HSCs at different time periods of culture (days 0, 0.5, 3 and 7); the images of HSCs in culture were captured at $\times 20$ and $\times 40$ magnifications using an Olympus FSX100 microscope. **Panels B** – mRNA expression of genes encoding extracellular matrix proteins (*Acta 2*, *Col1a1*) were determined by qRT-PCR for RNA samples obtained from freshly isolated HSCs (day 0) and HSCs cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively). **Panel C** – Total retinyl ester (RE) concentrations (ng/ng DNA) were determined by UPLC/MS/MS in lipid extracts of freshly

isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively); **Panel D** – mRNA expression of genes regulating retinoid metabolism (*Lrat*, *Cyp26A1*, *Rarb*) were determined by qRT-PCR in freshly isolated HSC (day 0) and HSCs cultured overnight, and for 3 and 7 days (day 0.5, 3 and 7 respectively). Values marked with different letters (a, b, c, d) are statistically different, $P < 0.05$. All values are given as the mean $\pm$ S.D., $n = 5$ for each time.

Figure 2. Total concentrations and acyl group composition of triglycerides and mRNA expression for some of their metabolizing enzymes during activation of primary mouse HSCs in culture on plastic.

Panel A – Total triglyceride (TG) concentrations (ng/ng DNA) were determined in lipid extracts of freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively). **Panel B** – Percentage of saturated, monounsaturated and polyunsaturated acyl groups in TGs were determined in freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days by UPLC/MS/MS in TLC-separated TGs after their saponification. **Panel C** – Heat map showing temporal changes in percentage (% TG)

of TG-derived fatty acyl species during HSCs activation in culture; heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to a percentage level indicated in the bottom scale bar. **Panel D** – Heat map showing temporal changes in percentage (% TG) of each TG-derived fatty acyl species with its range during HSCs activation in culture; heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to a percentage level indicated in the bottom scale bar; **Panels E and F**—mRNA expression of genes regulating triglyceride hydrolysis (*Atgl*, *Tgh1*, *Hsl*, *Lc3b*) and synthesis (*Dgat1*, *Dgat2*) were determined by qRT-PCR in freshly isolated HSC (day 0) and HSCs cultured overnight, and for 3 and 7 days (day 0.5, 3 and 7 respectively). Values marked with different letters (a, b, c, d) are statistically different, $P < 0.05$. All values are given as the mean $\pm$ S.D., $n = 5$ for each time point.

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Figure 3. Free fatty acid concentrations and mRNA expression for some of their metabolizing enzymes during activation of primary mouse HSCs in culture on plastic.

Panel A – Heat map showing temporal changes in concentrations (nmol/ng DNA) of total and all free fatty acids (FFA) determined by UPLC/MS/MS in lipid extracts of freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively); heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel B** – Heat map showing temporal changes in concentrations of each free fatty acid species

within its range during HSCs activation in culture; heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel C** – Temporal changes of total, palmitic (C16), arachidonic (C20:4) and docosahexaenoic (C22:6) free fatty acid concentrations during HSCs activation in culture; Fatty acid concentrations (nmol/ng DNA) are presented as mean $\pm$ SD, n = 4; * – significant difference compared to day 0 values, P<0.05; **Panel D** – Gene expression values for fatty acid desaturates (*Scd1*, *Scd2*) and elongases (*Elovl5*, *Elovl6*) were determined by qRT-PCR for RNA obtained from freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively). Values marked with different letters (a, b, c) are statistically different, P < 0.05. All values are given as the mean $\pm$ S.D., n = 5 for each time point.

Figure 4. Phosphatidylcholine concentrations during activation of primary mouse HSC in culture.

Panel A – a heat map showing temporal changes in concentrations (nmol/ng DNA) of total and individual phosphatidylcholine (PC) species determined by UPLC/MS/MS in lipid extracts of freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively); heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel B** – Heat map showing temporal changes of each phosphatidylcholine

species within its concentration range during HSC activation in culture; heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar.; **Panel C** – Temporal changes of total, 34:2, 36:6 and 38:4 phosphatidylcholine concentrations during HSC activation in culture; phosphatidylcholine concentrations (nmol/ng DNA) are presented as mean $\pm$ SD, n = 4; “*” - indicates a significant difference compared to day 0 values, P<0.05.

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Figure 5. Endocannabinoid, N-acylethanolamide concentrations and mRNA expression for some of their metabolizing enzymes during culture activation of primary mouse HSCs.

Panel A – Heat map showing temporal changes in concentrations of endocannabinoid and NAE species (pg/ngDNA) determined by UPLC/MS/MS in lipid extracts of freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 days respectively); heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel B** – Heat map showing temporal changes of each endocannabinoid and NAE species within its concentration range during HSC activation in culture; heat maps were generated in Excel

using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel C** – temporal changes of 2-arachidonoylglycerol (2-AG), arachidonylethanolamide (AEA) and palmitoylethanolamide (PEA) concentrations during HSC activation in culture; endocannabinoid concentrations (pg/ng DNA) are presented as mean $\pm$ SD, n=4; * – significant difference compared to day 0 values, P<0.05; **Panel D** – mRNA expression of genes encoding endocannabinoid- and NAE-metabolizing enzymes. Gene expression values were determined by qRT-PCR for RNA obtained from freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively). Values marked with different letters (a, b, c) are statistically different, P < 0.05. All values are given as the mean $\pm$ S.D., n = 5 for each time point.

Figure 6. Ceramide concentrations and ceramide-metabolizing enzymes mRNA expression during activation of primary mouse HSC in culture.

Panel A – Heat map showing temporal changes in concentrations of total and all the analyzed ceramide species determined by UPLC/MS/MS in lipid extracts of freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively); heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar. **Panel B** – Heat map showing temporal changes of each ceramide species within its concentration range during

HSC activation in culture; heat maps were generated in Excel using conditional formatting; intensities of green and red correspond to the concentration level indicated in the bottom scale bar.; **Panel C** – Temporal changes of total, N-palmitoyl (C16) and N-tetracosenoyl (C24:1) ceramide concentrations during HSC activation in culture; ceramide concentrations (pmol/ng DNA) are presented as mean $\pm$ SD, n = 4; * – significant difference compared to day 0 values, P<0.05; **Panel D** – mRNA expression of genes encoding ceramide metabolizing enzymes. Gene expression values were determined by qRT-PCR for RNA obtained from freshly isolated HSCs (day 0) and the cells cultured overnight, 3 and 7 days (day 0.5, 3 and 7 respectively). Values marked with different letters (a, b) are statistically different, P < 0.05. All values are given as the mean $\pm$ S.D., n = 5 for each time point.