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Identification and in vivo detection of side-chain hydroxylated metabolites of 4β-hydroxycholesterol

Martin Roumain, Owein Guillemot-Legris, Hafsa Ameraoui, Mireille Alhouayek, Giulio G. Muccioli *

Bioanalysis and Pharmacology of Bioactive Lipids Research Group, Louvain Drug Research Institute, Belgium

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ABSTRACT

Oxysterols are oxidized derivatives of cholesterol that are formed by enzymatic processes or through the action of reactive oxygen species. Several of these bioactive lipids have been shown to be affected and/or play a role in inflammatory processes. 4β-hydroxycholesterol is one of the major oxysterols in mice and humans and its levels are affected by inflammatory diseases. However, apart from its long half-life, little is known about its catabolism. By incubating 4β-hydroxycholesterol with mouse mitochondria-enriched liver fractions, as well as 25-hydroxycholesterol and 27-hydroxycholesterol with recombinant CYP3A4, we identified 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol as 4β-hydroxycholesterol metabolites. Supporting the biological relevance of this metabolism, we detected both metabolites after incubation of J774, primary mouse peritoneal macrophages and PMA-differentiated THP-1 cells with 4β-hydroxycholesterol. Across our experiments, the incubation of cells with lipopolysaccharides differentially affected the levels of the 25- and 27-hydroxylated metabolites of 4β-hydroxycholesterol. Finally, 4β,27-dihydroxycholesterol was also detected in mice liver and plasma after intraperitoneal administration of 4β-hydroxycholesterol. To our knowledge, this is the first report of the in vitro and in vivo detection and quantification of 4β-hydroxycholesterol metabolites.

1. Introduction

Oxysterols are oxidized derivatives of cholesterol. They are formed either by enzymatic processes, mainly involving cytochromes, or through the action of reactive oxygen species [1]. They have long been considered as mere metabolic intermediates of sterols' metabolic pathways such as bile acids or steroid hormones. However, in recent years, a growing number of studies support their active role in different pathophysiological processes, mainly related to immunity, inflammation and cancer [2–8]. They can thus be considered as *bona fide* bioactive lipids (or lipid mediators). Moreover, several oxysterols (e.g. 7β-hydroxycholesterol [9]; 25-hydroxycholesterol [10,11]; 27-hydroxycholesterol [4,11–13]; 24(*S*)-hydroxycholesterol [11,12]) have been proposed as disease biomarkers (e.g. in inflammatory diseases, in cancer, etc.).

The study of the oxysterome (i.e. the oxysterols, their enzymes and receptors) is no easy task as the metabolism of oxysterols is complex and intricate [14]. Indeed, several oxysterols are synthesized by more than one enzyme, and a given enzyme may produce several oxysterols. For instance, 7α-hydroxycholesterol is produced by CYP7A1 and is transformed into 7α,27-dihydroxycholesterol by CYP27A1. However, 7α,27-dihydroxycholesterol can also be synthesized by CYP7B1 from 27-hydroxycholesterol, which is also a product of CYP27A1 [14]. Thus, further studying the metabolism of oxysterols should allow for a better understanding of their physiological role and potential pharmacological interest. As a matter of fact, the effects reported for a given oxysterol can be due to its interaction with its target(s) but also to the actions of its potential metabolites.

4β-hydroxycholesterol is one of the most abundant circulating oxysterols in mice [14]. It is synthesized, from cholesterol, by the murine CYP3A11 and CYP3A13. In humans, CYP3A4 and CYP3A5 are the enzymes responsible for 4β-hydroxycholesterol synthesis. In human plasma 4β-hydroxycholesterol levels are reported to be around 10–60

Abbreviations: EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; KCZ, ketoconazole; LC-HRMS, liquid chromatography high-resolution mass spectrometry; LPS, lipopolysaccharides; NIC, nicardipine; OHC, hydroxycholesterol.

* Correspondence to: Bioanalysis and Pharmacology of Bioactive Lipids Research Group, Louvain Drug Research Institute, Université catholique de Louvain, Av. E. Mounier, 72 (B1.72.01), 1200 Brussels, Belgium.

E-mail address: giulio.muccioli@uclouvain.be (G.G. Muccioli).

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ng/ml [15,16]. We and others have found that 4β-hydroxycholesterol levels are decreased in inflammatory pathologies such as Crohn's disease [2] and obesity [17,18]. Surprisingly, apart from its long half-life of several days [19,20], little is known about its catabolism in vivo. One study suggested that 4β-hydroxycholesterol could be metabolized into 4β,27-dihydroxycholesterol, 4β,7-dihydroxycholesterol and 4β,24-dihydroxycholesterol by recombinant cytochromes, CYP27A1, CYP7A1 and CYP46, respectively [19]. However, this metabolism has not been demonstrated in intact cellular systems nor in vivo.

27-hydroxycholesterol and 25-hydroxycholesterol are two other well-studied oxysterols in the context of inflammation. 27-hydroxycholesterol is produced by the mitochondrial CYP27A1 [21–24] and is implicated in inflammatory conditions, such as atherosclerosis [4] and non-alcoholic steatohepatitis [25]. Similarly to 4β-hydroxycholesterol, its levels were also found to be altered in patients suffering from inflammatory bowel diseases [2]. 25-hydroxycholesterol is formed by the microsomal cholesterol 25-hydroxylase (CH25H) [26]. This oxysterol has also been identified as a product of CYP27A1 [27,28]. It has been shown to be involved in murine models of inflammatory pathologies such as inflammatory bowel diseases [29] and chronic obstructive pulmonary disease [3].

In this context, we decided to investigate further the catabolism of 4β-hydroxycholesterol, more specifically its potential 25-hydroxylation and 27-hydroxylation. Indeed, the effects of 4β-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol in inflammatory diseases may be due in part to their common metabolites. Thus, we designed a set of experiments allowing for the correct identification of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in the absence of commercially available analytical standards. First, we focused on the 25- and 27-hydroxylation of 4β-hydroxycholesterol by CYP27A1. Then, we assessed the 4β-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol. After identifying their common metabolites, we set out to evaluate the biological relevance of these pathways in mouse (J774 cells) and human (THP-1 cells) macrophage-like cell lines, in primary mouse peritoneal macrophages, and in mice.

2. Material and methods

2.1. Material

Sucrose, ethylenediaminetetraacetic acid (EDTA), Tris-HCl, dithiothreitol, NADPH, isocitrate dehydrogenase, trisodium isocitrate, sodium phosphate, (2-hydroxypropyl)-β-cyclodextrin, butylated hydroxytol uene (BHT), phorbol 12-myristate 13-acetate (PMA) and nicardipine hydrochloride were purchased from Sigma-Aldrich. *E. coli* O55:B5 lipopolysaccharides (LPS) was also purchased from Sigma-Aldrich. Ketoconazole was purchased from Fagron. Human recombinant CYP3A4, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and magnesium chloride were part of a Corning Supersomes enzymatic assay system. Human mitochondria-enriched liver fractions and human liver microsomes were purchased from XenoTech. Dichloromethane, methanol, *n*-hexane, isopropanol and silica were purchased from VWR. Water was bidistilled in-house. All materials were stored under the conditions specified by the manufacturers.

Cholesterol, 4β-hydroxycholesterol, 24(*S*)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, [25,26,26,26,27,27,27-$^2H_7$]4β-hydroxycholesterol (d_7-4β-hydroxycholesterol) and [25,26,26,26,27,27,27-$^2H_7$]24(*R/S*)-hydroxycholesterol (d_7-24-hydroxycholesterol) were purchased from Avanti Polar Lipids and stored as aliquots in methanol at − 80 °C.

2.2. Methods

2.2.1. Preparation of mitochondria-enriched fractions

Murine mitochondria-enriched liver fractions were obtained as described by Fernández-Vizarra [30]. Briefly, mouse liver tissue was homogenized in a homogenization medium containing 0.32 M sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.4). The tissue homogenate was centrifuged at 1000 × *g* for 5 min at 4 °C. Then, the supernatant was recovered and centrifuged at 15,000 × *g* for 2 min at 4 °C. The pellet was recovered and washed twice with the homogenization medium. Following resuspension in buffer and protein quantification, mitochondria-enriched liver fractions were then stored as aliquots at − 80 °C until their use.

Mitochondria-enriched fraction from J774 cells were prepared as described by Li [31]. Briefly, pelleted cells were resuspended and homogenized in a homogenization medium containing 0.25 M sucrose, 0.5 mM EDTA and 10 mM potassium phosphate (pH 7.4). The cell homogenate was centrifuged at 600 × *g* for 10 min at 4 °C. The supernatant was recovered and centrifuged at 6700 × *g* for 20 min at 4 °C. The pellet was then washed twice in the homogenization medium. Proteins were quantified and mitochondria-enriched fractions were stored as aliquots at − 80 °C until their use.

2.2.2. Mitochondria activity assay

The mitochondrial enzymatic activity was assessed using a protocol adapted from the literature [31–35]. Murine mitochondria-enriched fractions (500 μg of protein content) isolated from liver tissue or J774 cells as described above, or human mitochondria-enriched liver fractions (200 μg of protein content), were incubated at 37 °C for 20 min (or the timing specified in the legends of the figures) with EDTA (100 nmol), dithiothreitol (500 nmol), NADPH (600 nmol), isocitrate dehydrogenase (0.2 U) and trisodium isocitrate (2.5 μmol) in 500 μl sodium phosphate (100 mM, pH 7.5). The substrate, 40 nmol of cholesterol or 4β-hydroxycholesterol depending on the experiment, was added as a 10 μl solution containing (2-hydroxypropyl)-β-cyclodextrin 45 % (w/v) in water. The reaction was stopped by the addition of ice-cold dichloromethane and methanol used for the subsequent lipid extraction (see below for the oxysterol analysis). As an additional control, in each experiment we measured the oxysterol content in tubes where the mitochondria-enriched fraction was added after the addition of the organic solvents used to stop the reaction.

2.2.3. Recombinant CYP3A4 assay

Human recombinant CYP3A4 (20 pmol) prepared from baculovirus-transfected insect cells (obtained from Corning) were incubated at 37 °C for 60 min with $NADP^+$ (650 nmol), glucose-6-phosphate (1.65 μmol), glucose-6-phosphate dehydrogenase (0.2 U) and magnesium chloride (1.65 μmol) in 500 μl potassium phosphate (100 mM, pH 7.4), based on the manufacturer's protocol. The substrate, 100 nmol of 24(*S*)-hydroxycholesterol, 25-hydroxycholesterol or 27-hydroxycholesterol was added as a 10 μl solution containing (2-hydroxypropyl)-β-cyclodextrin 45 % (w/v) in water. Negative controls were prepared using the same cofactors and substrates incubated with microsomes prepared from insect cells transfected with control baculovirus not containing the CYP3A4. The reaction was stopped by the addition of ice-cold dichloromethane and methanol used for the subsequent lipid extraction (see below for the oxysterol analysis).

2.2.4. J774 cells culture and treatment

J774 cells were cultured in 175 mm^2 flasks under standard conditions (37 °C in a humidified 5 % CO_2 incubator) in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and antibiotics (100 μg/ml of streptomycin, 100 units/ml of penicillin). Cells were routinely passaged twice per week.

When assessing 4β-hydroxycholesterol catabolism, cells were seeded overnight into 100 mm dishes (10^7 cells per dish). Cells were then incubated with fresh culture medium (1 % FBS) containing 4β-hydroxycholesterol (10 μM) or vehicle (DMSO, 0.1 %), in the presence or absence of LPS (100 ng/ml), for 4 or 24 h. As (2-hydroxypropyl)-β-cyclodextrin is known to sequestrate hydrophobic molecules (e.g. cholesterol) and therefore impact the cellular lipid content [36–40], we

added 4β-hydroxycholesterol to the cells using DMSO, rather than the cyclodextrin, as previously performed [41]. At the end of the incubation, the media was removed, and the cells were scraped from the culture dish using 2 ml of ice-cold PBS-EDTA (7 mM). The content in oxysterol was then analyzed as described in Section 2.2.7.

When assessing mRNA and protein expression, cells were seeded overnight in 24-well plates (2.5×10^5 cells per well). Cells were then incubated with fresh culture medium in the presence or absence of LPS (100 ng/ml) for 8 h. At the end of the incubation, media was recovered for ELISA assessment of protein expression and TriPure (Roche) was added to the cells for later assessment of mRNA expression.

2.2.5. THP-1 cells culture and treatment

The human monocytic cell line THP-1 (ATCC, Manassas, VA, USA) was maintained in RPMI 1640 medium containing 10 % FBS and antibiotics (100 μg/ml of streptomycin, 100 units/ml of penicillin). Cells were cultivated in a humidified environment at 37 °C with 5 % CO_2.

To induce the differentiation into macrophages, THP-1 monocytes were seeded in complete medium at a density of 6×10^5 cells/ml in 10 mm dishes (9×10^6 cells per dish) and were treated with PMA (50 nM) for 48 h. The medium was then replaced with fresh medium and the cells kept in the incubator for an additional 24 h to complete differentiation [42].

When assessing 4β-hydroxycholesterol catabolism, THP-1-derived macrophages were incubated with fresh culture medium (1 % FBS) containing 4β-hydroxycholesterol (10 μM) or vehicle (DMSO, 0.1 %), in the presence or absence of LPS (100 ng/ml), for 24 h. At the end of the incubation, the media was recovered, and the cells were scraped from the culture dish using 2 ml of ice-cold PBS-EDTA (7 mM). The content in oxysterol was then analyzed as described in Section 2.2.7.

When assessing mRNA and protein expression, cells were seeded in 24-well plates (10^5 cells per well) and differentiated into macrophages as described above. THP-1-derived macrophages were then incubated with fresh culture medium in the presence or absence of LPS (100 ng/ml), for 8 h. At the end of the incubation, media was recovered for ELISA assessment of protein expression and TriPure was added to the cells for later assessment of mRNA expression.

2.2.6. Primary cells and in vivo studies

Studies involving mice were conducted in accordance with the EU Directive 2010/63/EU, transformed into the Belgian Law of May 29, 2013 regarding the protection of laboratory animals (lab agreement number LA1230365) and were approved by animal studies ethics committee of the health sector of the UCLouvain (2017/UCL/MD/024). Mice (from Janvier Labs) were housed in filter-top cages under controlled conditions of temperature (20–22 °C) and artificial light (12-h light-dark cycle), and supplied with drinking water and food *ad libitum*.

2.2.6.1. Primary peritoneal macrophage isolation. Naïve murine peritoneal macrophages were obtained by peritoneal lavage as previously described [43]. Briefly, C57BL/6 mice (35 females, 12–14 weeks old) were euthanized, then peritoneal cells were recovered by performing a peritoneal lavage using 5 ml ice-cold PBS-EDTA (0.5 mM). The cells in the peritoneal lavage were pelleted (5 min, 250 × *g*, 4 °C) and resuspended in culture media (RPMI supplemented with 10 % FBS (v/v), 100 μg/ml of streptomycin and 100 units/ml of penicillin). Cells were plated in 100 mm cell culture dishes (1.2×10^7 cells per dish) for oxysterol quantification or 12-well plates (10^6 cells per well) for RT-qPCR analysis. Three hours after the initial plating, the culture medium was removed, the cells were washed with PBS to remove non-adherent cells, and fresh medium was added to the cells. Sixteen hours later, cells were incubated with fresh culture medium (1 % FBS) containing 4β-hydroxycholesterol (10 μM) or vehicle (DMSO, 0.1 %), in the presence or absence of LPS (100 ng/ml), for 24 h (or 8 h for mRNA and protein expression). At the end of the incubation, the media was removed, and the cells were scraped from the culture dish using 2 ml of ice-cold PBS-EDTA (7 mM). The content in oxysterol was analyzed as described in Section 2.2.7.

2.2.6.2. 4β-hydroxycholesterol in vivo administration. Upon arrival, male mice (8–10 weeks old, C57BL/6 background) were randomly split into groups (with weight matching between the control and treated groups) of eight mice each. Mice wellbeing was monitored daily. 4β-hydroxycholesterol (10 mg/kg) was administered intraperitoneally in a mixture of saline–ethanol–Tween 80 (18:1:1, v/v/v), once every three days for three weeks. The dosing and administration pattern were selected based on its half-life and previous experiments [2,19]. Mice were anesthetized with isoflurane 48 h after the last administration, blood was recovered transcardially and the mice euthanized by cervical dislocation. The liver was recovered and snap-frozen in liquid nitrogen. All the samples were stored at − 80 °C until the analysis.

2.2.7. Oxysterol analysis by LC-HRMS

Oxysterols were analyzed using a validated LC-HRMS quantification method we previously described [44]. Briefly, samples (i.e. liver homogenates, plasma, reaction mixtures, scraped cells, culture media) were placed in glass vials containing deuterated internal standards (d_7-4β-OHC, d_7-24-OHC), dichloromethane, methanol (containing 10 μg BHT) and water (containing 20 ng EDTA) (4:2:1, v/v/v). Samples were then sonicated in ice-cold water for 10 min prior to centrifugation (10 min, 600 × *g*, 4 °C). Organic phases were recovered and dried under a stream of nitrogen. The lipid extracts were resuspended in dichloromethane and transferred on unmodified silica column for purification by solid-phase extraction. Cholesterol was eliminated with *n*-hexane/isopropanol (99:1, v/v) and oxysterols recovered with *n*-hexane/isopropanol (7:3, v/v). Eluates were recovered and dried under a stream of nitrogen. The resulting residues were resuspended in methanol and prepared for injection. As previously described, the LC-HRMS system consisted of an Accela LC system (Thermo Fisher Scientific) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) [41,45]. Chromatographic separation was performed using an Ascentis Express C18 column (150 × 4.6 mm; 2.7 μm, Sigma), kept at 15 °C. Mobile phase was a gradient of methanol and water containing acetic acid. Mass spectrometry analysis was performed using an atmospheric pressure chemical ionization source in the positive mode. Mass calibration was performed before each experiment. Raw data were processed with Xcalibur (Thermo Fisher Scientific).

As reported in the literature, oxysterols undergo in-source fragmentations during the mass spectrometry analysis [44,46–48]. Therefore, the pseudomolecular ions $[M + H]^+$ are poorly detected, if detected at all. This well-known phenomenon was taken into account during the analysis and dehydrated ions ($[M + H - H_2O]^+$ and/or $[M + H - 2H_2O]^+$) were used for quantification instead.

4β-hydroxycholesterol, 24(*S*)-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol were quantified using calibration curves of pure standards against their respective internal standard. For the estimated production of 4β-hydroxycholesterol metabolites, as authentic standards of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol are not available, the calibration curve obtained for 7α,27-dihydroxycholesterol, a structurally similar oxysterol, was used. As previously published, all calibration curves were subjected to the entire analytical process [44].

2.2.8. RNA extraction and real-time qPCR

RNA extraction and RT-qPCR were performed as previously described [49]. Briefly, total RNA was extracted from J774 cells, THP-1 cells and primary macrophages using the TriPure reagent according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (RT GoScript mix, Promega). The reaction involved

three steps: Primer annealing at 25 °C followed by DNA polymerization at 42 °C for 1 h and finally enzyme deactivation at 70 °C for 15 min. qPCR was performed with a with a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using the GoTaq qPCR Master Mix (Promega). qPCR included 45 amplification cycles consisting of the following steps: denaturation of the template cDNA at 95 °C for 3 s, annealing of the primers to the target template at 60 °C for 26 s and extension at 72 °C for 10 s. Products were analyzed by performing a melting curve at the end of the PCR. Data are normalized to mRNA expression of the 60S ribosomal protein L13 (*Rpl13*) for murine cells, and the 60S ribosomal protein L19 (*RPL19*) for human cells. Primer sequences for qPCR are listed in Table S1.

2.2.9. Statistical analysis

All data are presented as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism (version 9.0). Two-tailed Student's *t* test, or Mann-Whitney's test when relevant, were used to compare two groups of unpaired values. One-way ANOVA, or Kruskal-Wallis' test when relevant, coupled to Dunnett's or Dunn's test were used to compare more than two groups of unpaired values. Finally, a two-way ANOVA test, coupled to Tukey's test, was used when comparing groups containing two distinct categorical variables. Statistical significance was admitted when $p < 0.05$.

3. Results and discussion

3.1. 4β-hydroxycholesterol is metabolized by murine mitochondria-enriched liver fractions

We started this work by focusing on the 27-hydroxylation of 4β-hydroxycholesterol. As the mitochondrial enzyme CYP27A1 is responsible for the in vivo formation of 27-hydroxycholesterol, we set up an assay to measure its activity [21,22,24]. For this, we prepared mitochondria-enriched liver fractions from C57BL/6 mice, as mitochondrial fractions from the liver are known to have a strong CYP27A1 activity [23,31]. To control for the quality of the preparation, we incubated the murine mitochondria-enriched liver fractions with cholesterol and quantified the produced oxysterols by LC-HRMS. As expected using a mitochondrial fraction, we did not observe any production of 4β-hydroxycholesterol, which is produced by the microsomal enzymes CYP3A11/13 (data not shown). However, our data show a production of 27-hydroxycholesterol, that was absent when the preparation was inactivated using methanol, thus confirming the 27-hydroxylating activity of our preparation (Fig. 1A). To ascribe further this activity to CYP27A1, we used two structurally unrelated inhibitors of CYP27A1, namely nicardipine and ketoconazole [50,51]. 27-hydroxycholesterol production was blocked by nicardipine (87.2 % inhibition when used at 100 μM) and ketoconazole (89.3 % inhibition when used at 50 μM) supporting the involvement of CYP27A1 in the 27-hydroxylation of cholesterol (Fig. 1A). Besides 27-hydroxycholesterol, the LC-HRMS analysis of the samples revealed the presence of 25-hydroxycholesterol and 24(*S*)-hydroxycholesterol. However, this production was around 6 times lower compared to the production of 27-hydroxycholesterol (Table S2). The production of 25-hydroxycholesterol and 24(*S*)-hydroxycholesterol by the mitochondrial fraction could be explained by the already described 25- and 24(*S*)-hydroxylation activity of CYP27A1 [27]. This is further supported by the decreased production of 25-hydroxycholesterol and 24(*S*)-hydroxycholesterol in the presence of the inhibitors (Fig. 1A).

After validating our assay using cholesterol as a substrate, we applied it to 4β-hydroxycholesterol and looked for its potential primary mitochondrial metabolites. Thus, following the incubation of 4β-hydroxycholesterol with the mitochondria-enriched liver fractions, we analyzed the lipid products of the reaction by LC-HRMS. We observed several peaks on the chromatogram which were absent when an inactivated mitochondrial fraction was used (Fig. 1B). Interestingly, the production of three of these peaks – namely peaks 1, 2, and 3 – was significantly decreased when 4β-hydroxycholesterol was incubated with the mitochondrial fraction in the presence of nicardipine and ketoconazole (60.3–84.9 % inhibition) (Fig. 1C). Further supporting their enzymatic production, we observed that the signal of these peaks increases in a time-dependent manner with the incubation time (Fig. 1D). Of note, consistent with what is known on oxysterol analysis, these peaks were not detected in the *n*-hexane-isopropanol 99:1 (v/v) fraction of the solid-phase extraction, but were instead completely recovered in the *n*-hexane-isopropanol 7:3 (v/v) fraction, together with the other oxysterols (data not shown) [44,52,53].

Given their production in the presence of 4β-hydroxycholesterol, which is impaired by CYP27A1 inhibitors, peaks 1, 2, and 3 could be metabolites of 4β-hydroxycholesterol produced by this enzyme. Their *m/z* ratio, obtained using a high-resolution mass spectrometer (Fig. S1), and their retention times typical of dihydroxylated cholesterol, as well as the data we obtained when using cholesterol as substrate, led us to hypothesize that the observed peaks are 4β,25-dihydroxycholesterol, 4β,24(*S*)-dihydroxycholesterol and 4β,27-dihydroxycholesterol, respectively. In the absence of commercially available standards for these metabolites, we estimated their production by using the calibration curve of a structurally similar oxysterol, namely the 7α,27-dihydroxycholesterol. Using this approach, we estimated that peak 3 is produced in larger amounts compared to the other two (Table S2). We also observed a greater conversion of 4β-hydroxycholesterol compared to cholesterol into their respective metabolites (Table S2), which is in line with the results of Bodin et al. using recombinant CYP27A1 [19].

3.2. CYP3A4 is responsible for the 4β-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol

After discovering peaks of potential side-chain metabolites of 4β-hydroxycholesterol, we then sought to confirm their identity. We were unable to match their retention times with analytical standards as these are not commercially available. Thus, we opted for a biochemical approach, namely using the 4β-hydroxylating activity of CYP3A4 (the enzyme responsible for the 4β-hydroxylation of cholesterol in humans), to produce 4β-hydroxylated metabolites of several side-chain hydroxycholesterols that could be used to match the retention times and mass spectra with those of peaks 1, 2 and 3.

Therefore, we incubated 24(*S*)-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol with recombinant CYP3A4 and analyzed the resulting metabolites by LC-HRMS (Fig. 2). Based on the chromatograms and the retention times of the monohydroxylated and dihydroxylated oxysterols in this LC system, we determined that the retention times of 4β,25-dihydroxycholesterol, 4β,24(*S*)-dihydroxycholesterol and 4β,27-dihydroxycholesterol are 20.5 min, 21.0 min and 21.7 min, respectively (Fig. S2). When comparing the relative retention times (compared to d_7-24-hydroxycholesterol) of the different experiments, we found that peak 1 has the same relative retention time as 4β,25-dihydroxycholesterol (i.e. 0.79) and that peak 3 has the same relative retention time as 4β,27-dihydroxycholesterol (i.e. 0.84). Moreover, the high-resolution mass spectra of peaks 1 and 3 are matched in both experiments (Fig. S3A–B). These results support the identification of peak 1 and peak 3 as 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol, respectively (Fig. S2A–C). The relative retention time of peak 2 could not be reliably matched with any of the CYP3A4 metabolites of 24(*S*)-hydroxycholesterol (Fig. S2B), and therefore has not been properly identified. We also observed that 4β,27-dihydroxycholesterol seems to be produced in greater extent compared to 4β,25-dihydroxycholesterol and 4β,24(*S*)-dihydroxycholesterol (Fig. 2D).

Taken together, the data obtained using murine mitochondria-enriched liver fractions and recombinant CYP3A4 support the identification of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol as CYP27A1 metabolites of 4β-hydroxycholesterol.

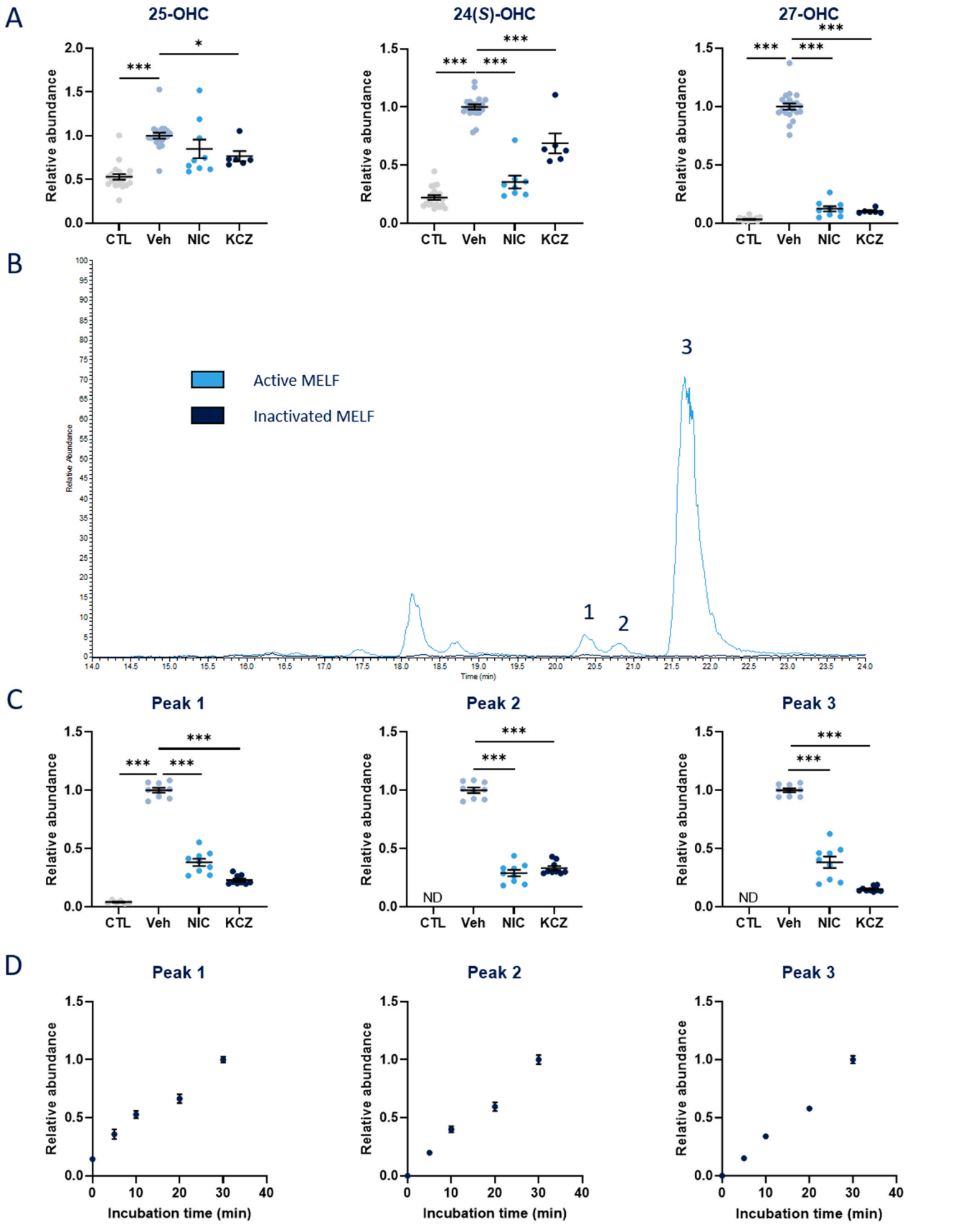

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Fig. 1. 4β-hydroxycholesterol is metabolized by murine mitochondria-enriched liver fractions. (**A**) Production of oxysterols after incubation of murine mitochondria-enriched liver fractions (mMELF, 500 μg of protein content) with cholesterol (40 nmol, 20 min). The effect of two CYP27A1 inhibitors (i.e. nicardipine (NIC, 100 μM) and ketoconazole (KCZ, 50 μM)) was assessed in comparison to the effect of vehicle (Veh, DMSO 0.1 %), set at 1. Basal oxysterol levels in the inactivated preparation were also measured (CTL). N = 2–7; n = 3. (**B**) Overlaid chromatograms obtained after incubation of 4β-OHC (40 nmol, 20 min) with mMELF. 4β-OHC was incubated before (blue) or after (black) protein inactivation. (**C**) Production of oxysterol metabolites following incubation of mMELF with 4β-OHC (40 nmol, 20 min). CYP27A1 inhibitors were used as described in (**A**). The effect of inhibitors is compared to the effect of vehicle (Veh, DMSO 0.1 %), set at 1. Basal oxysterol levels in the inactivated preparation were also measured (CTL). N = 3; n = 3. (**D**) Time-dependent production of 4β-OHC metabolites after incubation of mitochondria-enriched liver fractions with 4β-OHC (40 nmol) for 0 min, 5 min, 10 min, 20 min and 30 min, set at 1. N = 3; n = 3. OHC: hydroxycholesterol. ND: not detected. * $p < 0.05$; *** $p < 0.001$.

3.3. *Production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in the J774 cell line*

After the identification of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol, we sought to assess whether they can be detected in more complex biological matrices and whether they can be considered as *bona fide* metabolites of 4β-hydroxycholesterol.

As oxysterols have been suggested to modulate macrophage phenotype [54], we decided to start our investigations using a macrophage cell line. First, we incubated mitochondria-enriched fractions of the murine macrophage cell line J774 with cholesterol as substrate and measured a cholesterol 27-hydroxylase activity of 446.3 pmol min^{-1} mg^{-1}. Next, we incubated J774 cells with 4β-hydroxycholesterol and observed in the resulting LC-HRMS chromatograms peaks having retention times and mass spectra matching those of the previous experiments (Figs. S2–S3), thus confirming the production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in these cells. We then incubated J774 cells with 4β-hydroxycholesterol (or vehicle) during 4 or 24 h (Fig. 3A) and found a time-dependent increase of the signal corresponding to 4β,27-dihydroxycholesterol (Fig. 3B). In these experiments, 4β,25-dihydroxycholesterol was only detected in the 24 h incubation condition (data not shown). Then, we incubated these cells with 4β-hydroxycholesterol in the presence of ketoconazole, the CYP27A1 inhibitor with the stronger effect on 4β,27-dihydroxycholesterol production (see Fig. 1C). In these experiments we found a large decrease of the production of 4β,25-dihydroxycholesterol (70.8 % inhibition) and 4β,27-dihydroxycholesterol (84.9 % inhibition) in the presence of the CYP27A1 inhibitor (Fig. 3C). Of note, levels of 25-hydroxycholesterol and 27-hydroxycholesterol were also altered by ketoconazole treatment (Fig. S4A).

The proinflammatory activation of macrophages has a strong effect on their phenotype. Thus, we sought to investigate whether this would also affect the production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol. One of the most commonly used methods to activate macrophages is to use toll-like receptor 4 ligands such as LPS. In our previous works, 100 ng/ml of LPS induced a robust activation of the cells as assessed by the increased expression of pro-inflammatory markers [55,56]. Here we obtained similar results with increased secretion of IL-6 and TNFα (Fig. S4B). As reported in Fig. 3D, the cells produced both 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in the presence of 4β-hydroxycholesterol. Interestingly, this production was not affected by the presence of LPS. This is consistent

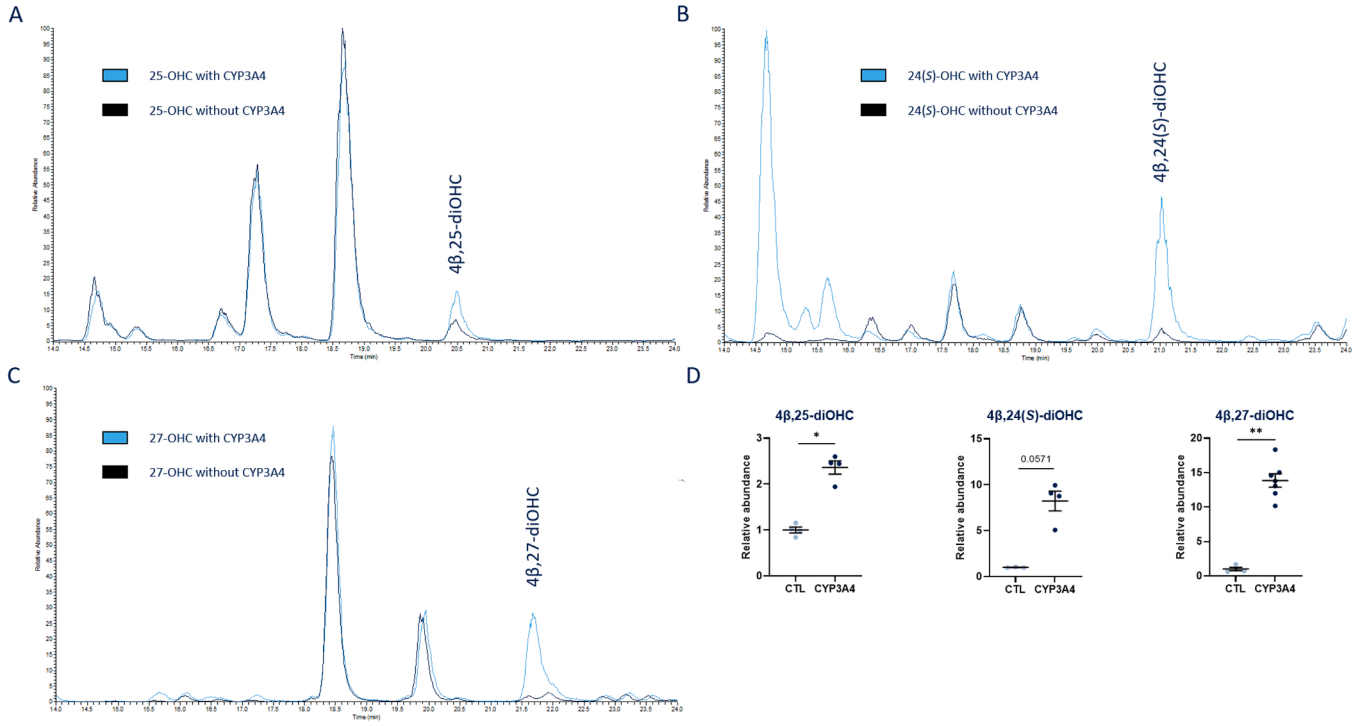

Fig. 2. CYP3A4 is responsible for the 4β-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol. Representative chromatograms showing the production of oxysterol metabolites after 60 min incubation of CYP3A4 with 100 nmol of 25-OHC (**A**), 24(*S*)-OHC (**B**) or 27-OHC (**C**). Substrates were incubated with human recombinant CYP3A4 from baculovirus-transfected insect cells (blue) or with preparation from insect cells infected with baculovirus not containing CYP3A4 (black). Ions were detected using an *m/z* ratio of 383.33090 for the metabolites of 25-OHC and 24(S)-OHC, and an *m/z* ratio of 401.34116 was used for the metabolites of 27-OHC. Mass tolerance was set at 5 mmu. Metabolites of 25-OHC and 27-OHC have been matched with peaks 1 and 3 from Fig. 1, respectively, and therefore have been identified as 4β,25-diOHC and 4β,27-diOHC. No CYP3A4 metabolite of 24(*S*)-hydroxycholesterol could be successfully linked to the metabolites of 4β-OHC identified in mMELF. (**D**) Relative abundance of CYP3A4 metabolites of 4β-OHC incubated with CYP3A4 or without CYP3A4 (CTL, set at 1). N = 2; n = 1–3 for 4β,25-diOHC and 4β,24(*S*)-diOHC measurements; N = 3; n = 1–3 for 4β,27-diOHC measurements. OHC: hydroxycholesterol. * $p < 0.05$; ** $p < 0.01$.

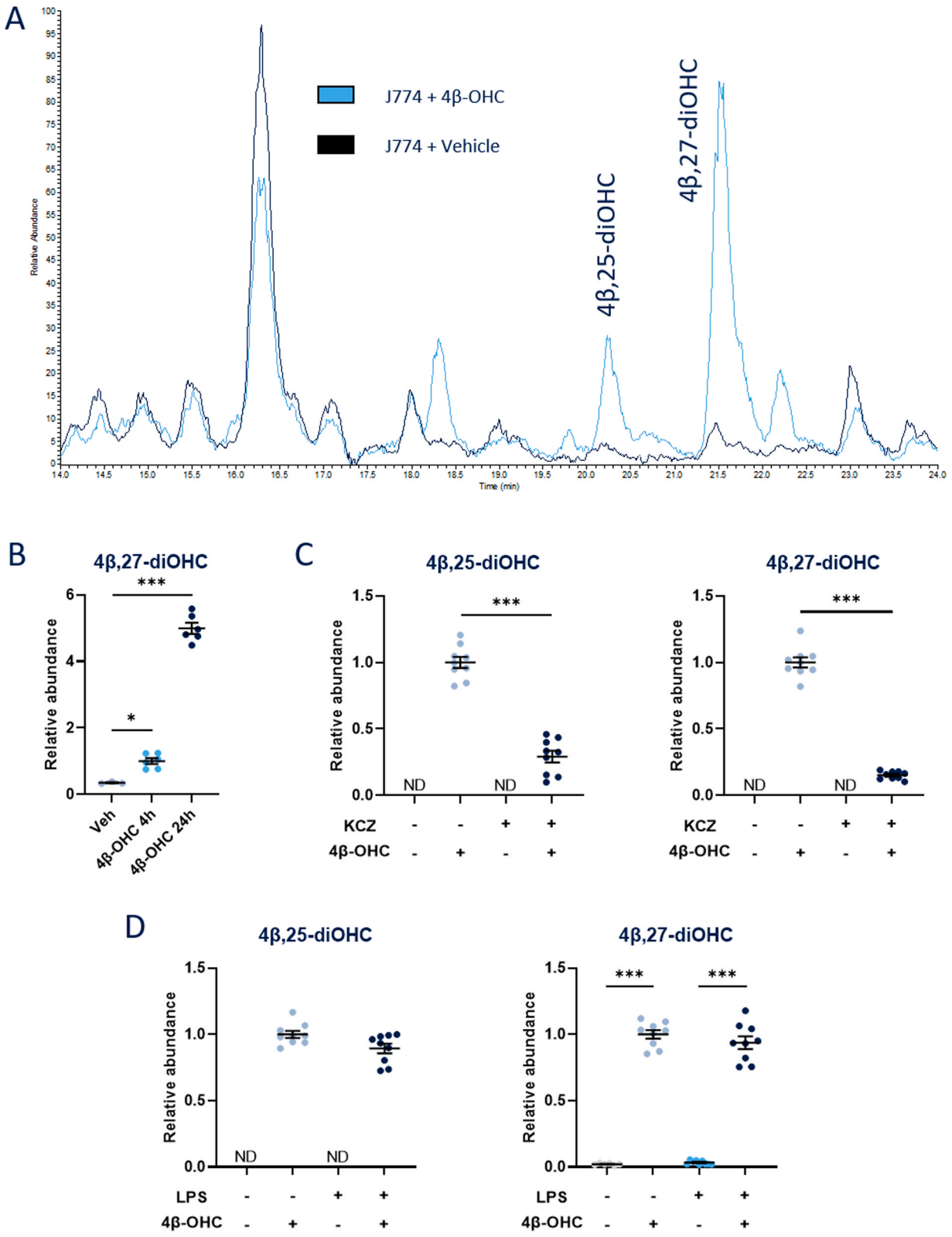

Fig. 3. Production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in the J774 cell line. (**A**) Representative overlaid chromatograms obtained after 24 h incubation of J774 cells (1×10^7) with 10 μM of 4β-OHC (blue) or with vehicle (black). (**B**) Relative abundance of 4β,27-diOHC after incubation of J774 cells in the presence of 4β-OHC (10 μM, 4 h (set at 1) or 24 h) or vehicle (Veh, DMSO 0.1 %). N = 2; n = 3. (**C**) Relative abundance of 4β,25-diOHC and 4β,27-diOHC after 24 h incubation of J774 cells (2×10^7) with 4β-OHC (10 μM) or with vehicle (DMSO 0.1 %) in the presence of ketoconazole (KCZ, 5 μM) or vehicle (DMSO 0.1 %). Data are expressed compared to the production of the metabolite in the presence of 4β-OHC and absence of KCZ, set at 1. N = 3; n = 3. (**D**) LPS incubation (100 ng/ml, 24 h) of J774 cells (2×10^7) did not affect the levels of 4β,25-diOHC and 4β,27-diOHC measured in the presence of 4β-OHC (10 μM). Data are expressed compared to the production of the metabolite in the presence of 4β-OHC and absence of LPS, set at 1. N = 3; n = 3. OHC: hydroxycholesterol. ND: not detected. *** $p < 0.001$.

with the absence of effect of LPS treatment on the mRNA expression of *Ch25h* and *Cyp27a1* (Fig. S4C). The absence of effect of LPS on *Ch25h* expression is consistent with the fact that in these conditions LPS did not increase the expression of *Ifng* (Fig. S4B) which is a known inducer of *Ch25h* expression. Moreover, *Cyp27a1* expression seems higher than *Ch25h* (Fig. S4C), which could explain the decrease of 4β,25-dihydroxycholesterol levels following ketoconazole treatment (Fig. 3C). Indeed, in these cells, CYP27A1 could have a significant impact on the production of this oxysterol.

3.4. LPS alters 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol levels in primary mouse macrophages

Based on the interesting results we obtained with the J774 cells, we decided to assess the production of these 4β-hydroxycholesterol metabolites by primary macrophages. We recovered resident peritoneal macrophages from 35 mice and investigated the production of 4β-hydroxycholesterol metabolites in the presence of LPS, which markedly activated these cells, as illustrated by the increased secretion of IL-6 and

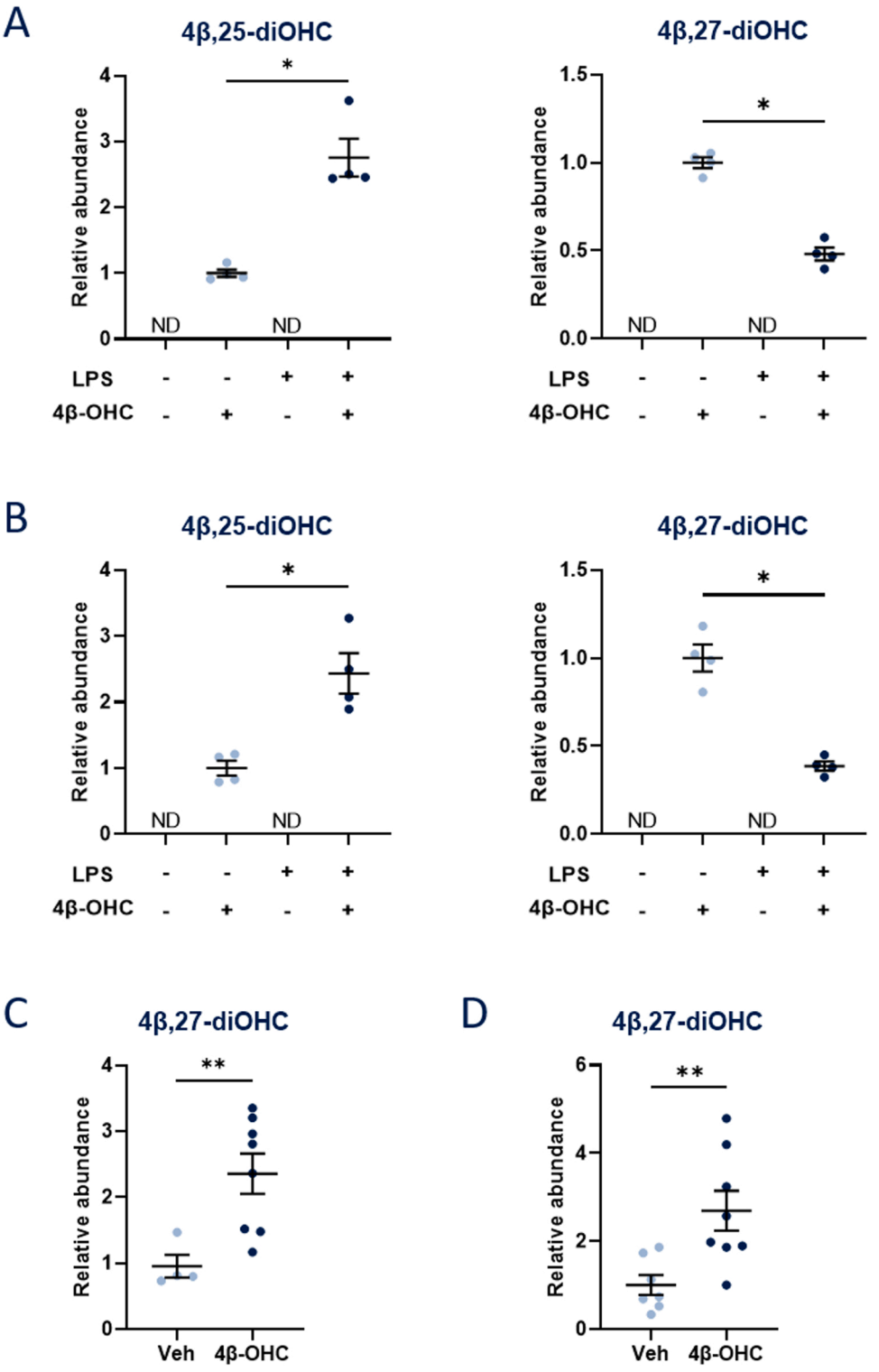

Fig. 4. Production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in primary mouse macrophages and in vivo. LPS incubation (100 ng/ml, 24 h) of primary mouse peritoneal macrophages alters the levels of 4β,25-diOHC and 4β,27-diOHC measured in the presence of 4β-OHC (10 μM) in cells (**A**) as well as in cell media (**B**). Data are expressed compared to the production of the metabolite in the presence of 4β-OHC and absence of LPS, set at 1. N = 1; n = 4 from pooled macrophages isolated from 35 C57BL/6 female mice (12–14 weeks old). (**C-D**) Relative abundance of 4β,27-diOHC measured in the liver (**C**) and plasma (**D**) from mice treated with 4β-OHC (10 mg/kg, i.p., every 3 days for 3 weeks) or vehicle (saline–ethanol–Tween 80 (18:1:1, v/v/v), Veh), set at 1. N = 8. OHC: hydroxycholesterol. ND: not detected. * $p < 0.05$; ** $p < 0.01$.

TNFα in the medium (Fig. S5A). We did not detect 4β,25-dihydroxycholesterol nor 4β,27-dihydroxycholesterol in primary macrophages incubated with vehicle. However, we did detect these metabolites in cells, as well as in culture media, after incubation with 4β-hydroxycholesterol (Fig. 4A–B). 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol levels were respectively 1.05- and 1.92-fold more detected in culture media compared to cell levels, highlighting the secretion of these metabolites. This secretion could enable these compounds to act as lipid mediators. In primary macrophages we found that 4β,25-dihydroxycholesterol production is increased after activating macrophages with LPS, which is consistent with the increased mRNA expression of *Ch25h* measured after 8 h of incubation (Fig. S5B). This fits with the increased expression of *Ifng* in the presence of LPS (Fig. S5A). On the other hand, 4β,27-dihydroxycholesterol is decreased in the presence of LPS, in line with the mRNA expression of *Cyp27a1* measured after 8 h of incubation with LPS (Fig. S5B). These results, combined with the results from the first set of experiments, support the role of CYP27A1 in the production of 4β,27-dihydroxycholesterol and suggest the involvement of CH25H, along with CYP27A1, in the production of 4β,25-dihydroxycholesterol.

Besides macrophages, we investigated the metabolism of 4β-hydroxycholesterol in vivo, focusing on 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol. Thus, we treated mice during 3 weeks with intraperitoneal administration of 4β-hydroxycholesterol (or vehicle) once every 3 days, based on its reported half-life [19,20]. We first looked in the liver as it is expected to be the primary site of metabolism. Quite interestingly, we detected an estimated 0.98 ± 0.18 pmol/g of 4β,27-dihydroxycholesterol in vehicle-treated mice (based on 7α,27-dihydroxycholesterol calibration curve) and found that its levels were increased upon administration of 4β-hydroxycholesterol (Fig. 4C). 4β,25-dihydroxycholesterol was not detected regardless of the treatment conditions. We also assessed the presence of 4β,27-dihydroxycholesterol in the plasma of these mice (estimated 3.35 ± 0.76 pmol/ml) and again found increased levels upon 4β-hydroxycholesterol treatment (Fig. 4D).

3.5. 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol are produced in human subcellular fractions and human differentiated THP-1 cells

Next, we sought to evaluate the production of these 4β-hydroxycholesterol metabolites in human subcellular fractions. Thus, we incubated 4β-hydroxycholesterol with mitochondria-enriched liver fractions isolated from human donors, similarly to our experiments with murine mitochondria-enriched liver fractions. Comparably to what we have found with murine samples, and in line with the subcellular localizations of CYP27A1 and CH25H [26], we detected a higher production of 4β,27-dihydroxycholesterol compared to 4β,25-dihydroxycholesterol (Table S2). Their levels were affected by nicardipine (100 μM) and ketoconazole (50 μM), which is consistent with an involvement of CYP27A1 (89.5–98.4 % inhibition) (Fig. 5A). Moreover, to further highlight the role of CYP27A1 in their production, we performed the same experiment using human liver microsomes (Fig. 5B). Indeed, while 27-hydroxylation activity is expected in mitochondria due to the presence of CYP27A1, it is not in human liver microsomes [57]. Although CYP27A1 inhibitors affect 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol levels to the same extent as in mitochondria (78.2–100.8 % inhibition), it is important to note that the productions observed in human liver microsomes are significantly lower (2.2–10.6 times) than in human mitochondria-enriched liver fractions (Table S2). While this does not rule out the possibility that other enzymes 25- or 27-hydroxylate 4β-hydroxycholesterol, it strongly suggests the predominance of CYP27A1 in the production of these metabolites in our experiments.

To assess further the translational value of our results, we investigated whether human macrophage-like cells could produce 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol and whether

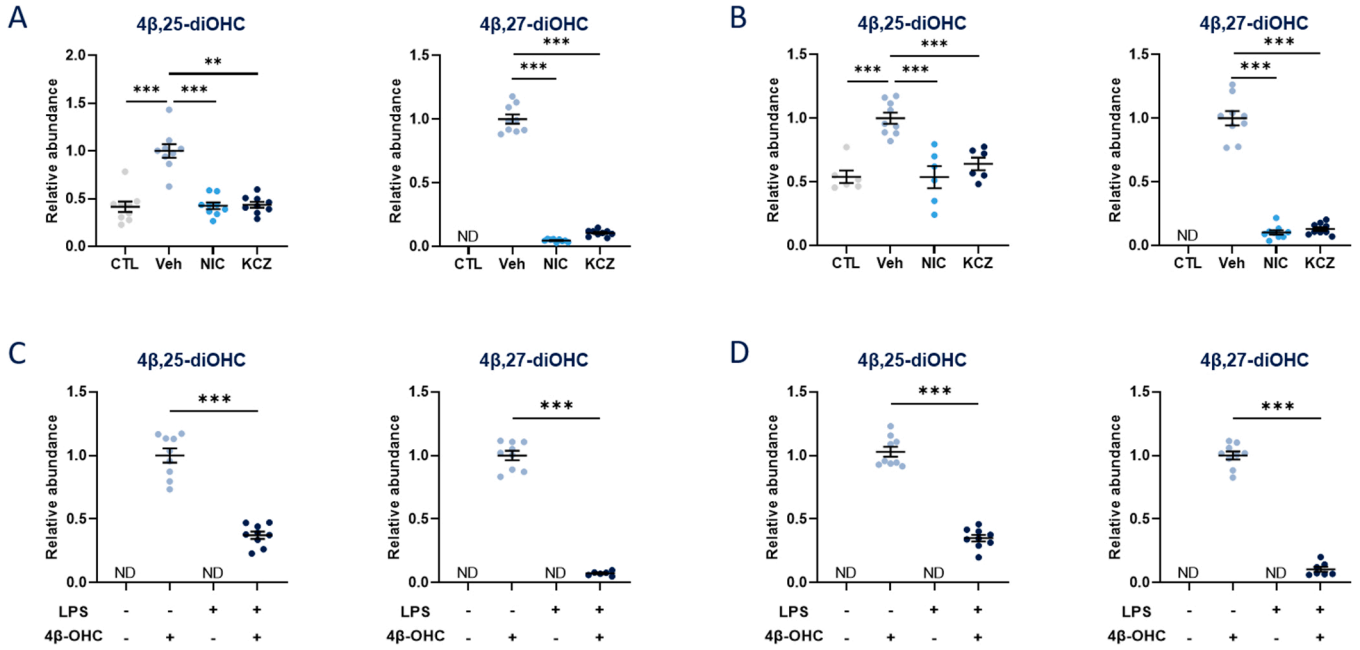

Fig. 5. Production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol in human mitochondria-enriched liver fractions, human liver microsomes and differentiated THP-1. Production of oxysterol metabolites following incubation of human mitochondria-enriched liver fractions (hMELF, 200 μg of protein content) (**A**) or human liver microsomes (hLM, 200 μg of protein content) (**B**) with 4β-OHC (40 nmol, 20 min). The effect of two CYP27A1 inhibitors (i.e. nicardipine (NIC, 100 μM) and ketoconazole (KCZ, 50 μM)) was assessed in comparison to the effect of vehicle (Veh, DMSO 0.1 %), set at 1. Basal oxysterol levels in the inactivated preparation were also measured (CTL). 4β,25-diOHC and 4β,27-diOHC are 2.2-fold and 10.6-fold more produced in hMELF compared to hLM, respectively. N = 3; n = 3. Relative abundance of 4β,25-diOHC and 4β,27-diOHC after 24 h incubation of differentiated THP-1 cells (2×10^7) (**C**) or cell media (**D**) with 4β-OHC (10 μM) or with vehicle (DMSO 0.1 %) in the presence of LPS (100 ng/ml). Data are expressed compared to the production of the metabolite in the presence of 4β-OHC and absence of LPS, set at 1. N = 3; n = 3. OHC: hydroxycholesterol. ND: not detected. *** $p < 0.001$.

incubation in the presence of LPS could affect their production. As shown in Fig. 5C, PMA-differentiated THP-1 cells produce both metabolites in the presence of 4β-hydroxycholesterol. Similarly to what we found for J774 and primary macrophages, cells incubated with LPS secrete more IL-6 and TNFα (Fig. S6C). Of note, we did not detect any mRNA expression of *IFNg* in the different condition tested. Contrasting with our experiments on murine cells, both 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol levels are decreased by LPS (Fig. 5C). This trend is also reflected in the cell media (Fig. 5D). Although the levels of 25-hydroxycholesterol and 27-hydroxycholesterol are also decreased by LPS in THP-1 cells and media, surprisingly, these results are not in line with the mRNA expression of *CH25H* and *CYP27A1* (Fig. S6D–F). Given that the levels of 25-hydroxycholesterol and 27-hydroxycholesterol, two oxysterols predominantly formed by CH25H and CYP27A1 respectively, are similarly affected as those of 4β-hydroxycholesterol metabolites, we hypothesize that LPS induces either a decreased activity of these enzymes, or an increased catabolism of side-chain oxysterols under these conditions.

4. Conclusions

In this paper, we report our findings on the metabolism of 4β-hydroxycholesterol. The combined use of *Cyp27a1*-expressing subcellular fractions and of recombinant CYP3A4, together with LC-HRMS analysis allowed us to successfully identify two of its metabolites, namely 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol, in the absence of commercially available standards (Table S3). As biochemical conditions are not always reminiscent of the complex environment found in intact biological systems, we also demonstrated that 25- and 27-hydroxylations are relevant metabolic pathways for 4β-hydroxycholesterol in murine and human macrophages (Table S4). This is also true in vivo, at least in mice, where we detected a significant production of 4β,27-dihydroxycholesterol following the administration of 4β-hydroxycholesterol. From an analytical perspective, the peaks were matched regarding their mass spectra and retention times across our different in vitro and in vivo experiments (Figs. S2–S3), further supporting the correct identification of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol.

Our results show that 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol can be produced by CYP3A4, one of the most important human enzymes in xenobiotic metabolism. This study also demonstrates that these potentially bioactive lipids are mitochondrial products of CYP27A1, through the use of CYP27A1 inhibitors in our in vitro and ex vivo experiments (Fig. 6). Moreover, the significant differences observed in the production of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol between human mitochondria-enriched liver fractions and human liver microsomes, as well as the associations between *Cyp27a1* expression and 4β,27-dihydroxycholesterol in murine cells further demonstrate the implication of CYP27A1 in the metabolism of 4β-hydroxycholesterol. Additionally, *Ch25h* expression has also been associated with 4β,25-dihydroxycholesterol levels, suggesting the implication of this enzyme in the production of the 4β-hydroxycholesterol metabolite.

Interestingly, we observed differences in the effect of LPS on the production of 4β-hydroxycholesterol metabolites across murine J774 cells, murine primary peritoneal macrophages and human differentiated THP-1 cells. This can be explained in part by the control exerted by IFNγ on *Ch25h* expression [58]. Indeed, *Ch25h* is known to be an interferon-stimulated gene and its expression is correlated with *Ifng* expression in our experiments on primary peritoneal macrophages ($r = 0.895$). The absence of an LPS-induced increase in IFNγ mRNA expression in J774 and THP-1 could explain why 4β,25-dihydroxycholesterol levels are not increased in those cell lines contrary to the primary macrophages. Moreover, IFNγ is also known to decrease

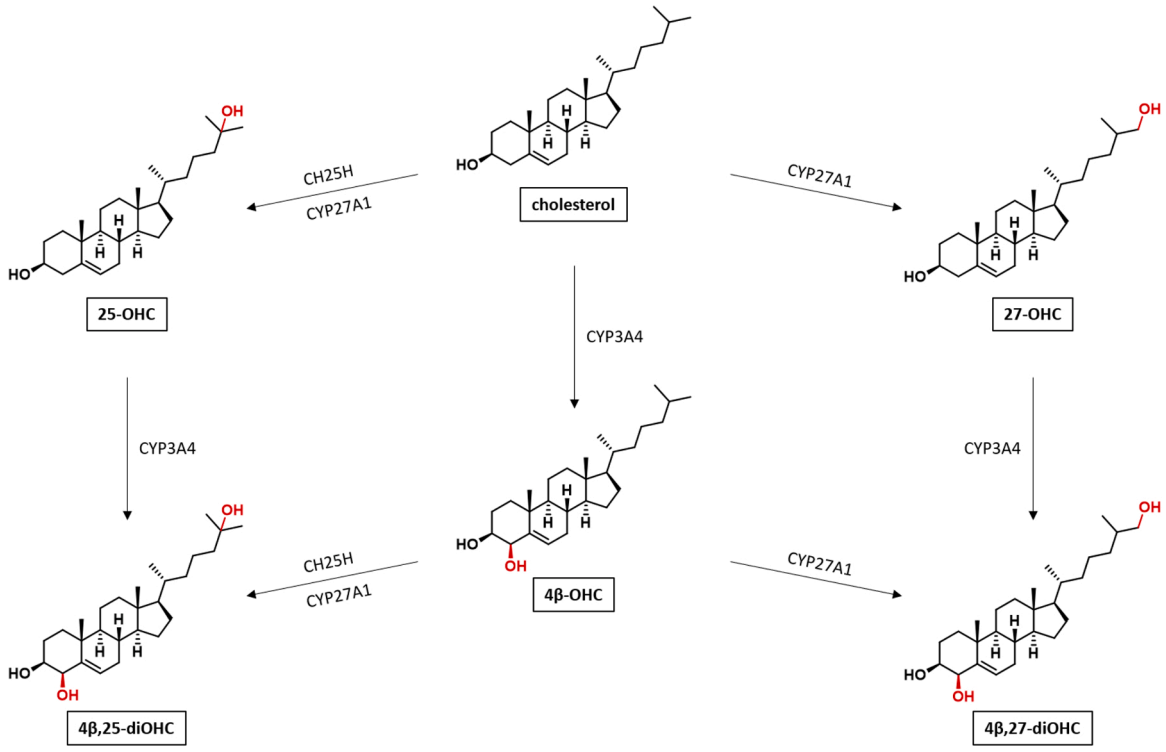

Fig. 6. Enzymatic oxysterol metabolism assessed in this study. 4β-hydroxycholesterol (4β-OHC) is formed by human CYP3A4 and CYP3A5 or their murine orthologues CYP3A11 and CYP3A13. Cholesterol 25-hydroxylase (CH25H) is considered the main enzyme producing 25-hydroxycholesterol (25-OHC) although it can also be formed by CYP27A1, CYP3A4 and ROS. 27-hydroxycholesterol (27-OHC) is formed from cholesterol by CYP27A1. In this report, we provide evidence showing the production of 4β,25-dihydroxycholesterol (4β,25-diOHC) and 4β,27-dihydroxycholesterol (4β,27-diOHC) by CYP3A4 using 25-OHC and 27-OHC as substrates. We also put forth the production of 4β,27-diOHC by CYP27A1 and the production of 4β,25-diOHC by CH25H and CYP27A1.

Cyp27a1 expression in macrophages [59]. This could explain the decrease of 4β,27-dihydroxycholesterol production in primary peritoneal macrophages.

In this study, we have shown that these newly discovered oxysterols are secreted into cell media and that 4β,27-dihydroxycholesterol is detected in mouse plasma. Thus, although further studies are needed to prove it, these lipids could behave as bioactive lipids. In this context, the availability of pure standards of 4β,25-dihydroxycholesterol and 4β,27-dihydroxycholesterol would help setting up highly sensitive quantification methods and assess their potential role in conditions such as cancer and inflammatory diseases. In addition, assessing the activity of these two metabolites on the main molecular targets of the oxysterols will be a priority once pure standards will be available.

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Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106376.

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