The nutrigenomic metabolite L-carnitine directly modulates the activity and expression of nuclear receptors in adipocytes, liver and muscle cells

Lorenz Förster
Medizinische Universität Wien
Dominic Indra
Medizinische Universitat Wien
Reinhold Hofbauer (reinhold.hofbauer@univie.ac.at)
Medizinische Universität und Universität Wien https://orcid.org/0000-0001-7837-035X

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Abstract

Background: L-carnitine is an indispensable metabolite in eukaryotic cells, which facilitates transport of long-chain fatty acids into the mitochondrial matrix for subsequent β-oxidation and helps to safeguard the acetyl-CoA level. Additionally, L-carnitine has been proven to exert a nutrigenomic effect, modulating the expression of numerous target genes. However, the diverging time-dependent effects of short-term and extended L-carnitine supplementation have not been investigated in more detail yet, especially in the interplay of adipocytes, liver and muscle cells. A cell culture model with conditions of L-carnitine deficiency and supplementation for these cell types was established to investigate the effects of L-carnitine on key nuclear receptors and their pathways.

Results: L-carnitine deficiency as well as L-carnitine supplementation to hepatocytes modulated protein activity of multiple nuclear receptor pathways (RAR, RXR, VDR, PPAR, HNF4, ER, LXR). On the transcriptional level, short-term L-carnitine supplementation initially exerted an inhibitory effect on the steady state mRNA levels of PPAR-α, PPAR-δ, PPAR-γ, RAR-β , LXR-α and RXR-α in adipocytes, liver and muscle cells. However, extended L-carnitine supplementation for 24 and 48 hours led to a significant upregulation of PPAR-α and PPAR-δ , being key regulators of lipid catabolism, thereby promoting lipolysis and β-oxidation. In addition, significant differences in transcriptional modulation were found between adipocytes, liver and muscle cells. Extended L-carnitine administration to hepatocytes also modulated mRNA expression levels of nuclear receptor target genes CYP2R1 , ALDH1A1 , HSD11B2 , OGT and HMGCR.

Conclusions: These findings show a clear nutrigenomic effect of L-carnitine on the protein activity and expression levels of selected nuclear receptors in different tissues, promoting lipolytic gene expression as well as decreasing transcription of adipogenic and insulin-resistance linked genes. Therefore L-carnitine supplementation obviously is a promising strategy supporting established antihyperlipidemic therapies.

Background

Lcarnitine (L3hydroxy4N–trimethylaminobutyrate) is a quaternary ammonium compound and an indispensable metabolite in all eukaryotic cells. Lcarnitine is synthesized from the essential amino acids lysine and methionine, with the highest rate of biosynthesis in the kidney and liver [1]. *Lca*rnitine has a number of essential roles for the metabolism, including transport of activated longchain fatty acids into the mitochondrial matrix for subsequent βoxidation as well as modulation of the acylCoA/CoA ratio in the cell [2]. Lcarnitine itself has also been proven to regulate the expression of numerous important genes for cellular metabolism and has therefore been coined as a nutrigenomic factor [3].

Systematic reviews indicate a significant beneficial impact of Lcarnitine on cardiovascular and metabolic diseases [4, 5]. Several studies show that exogenous carnitine administration is associated with a reduction of hyperlipidemia, hypertension, hyperglycemia and insulin resistance [6]. Additionally, Lcarnitine seems to have beneficial impacts on ventricular dysfunction, arrythmia and angina pectoris [7].

Although, as described, multiple lines of evidence suggest that Lcarnitine plays an important role in modulating metabolic functions in humans, few studies have elucidated the regulatory role of Lcarnitine on the molecular level. Artificial carnitine depletion *in vitro* has been shown to decrease mRNA expression of carnitine acetyltransferase (CRAT) and carnitine palmitoyl transferases (CPT1A and CPT2), whereas following carnitine supplementation leads to a full reversal of the downregulation [8]. In addition to this effect, a close connection between Lcarnitine and the peroxisome proliferatoractivated receptor system (PPAR) seems to exist. In adipocytes, supplementation of Lcarnitine suppresses the expression of *PPARγ,* which is involved in adipogenesis, and induces lipolytic gene expression [9]. Supplementation of Lcarnitine to hepatocytes seems to upregulate *PPARα* expression, which participates in lipid catabolism, as well as PPARαregulated genes such as CPT1 and acylcoenzyme A oxidase (ACOX) [10].

However, the diverging shortterm and longterm effects of Lcarnitine supplementation have not been investigated in more detail yet, especially in the interplay of adipocytes, liver and muscle cells. The major goal of this study was to investigate the regulatory mechanisms of Lcarnitine on nuclear receptor expression and activity. The nuclear hormone receptor superfamily of ligandactivated transcription factors represents the most important group of cellular nutrient sensors, enabling the organism to rapidly adapt to metabolic changes by inducing appropriate genes and pathways [11, 12]. We hypothesized that Lcarnitine has distinct time-dependent effects on nuclear receptor activity and expression. Therefore, the effects on the protein activity of subfamily I (RAR, LXR, VDR, PPAR), subfamily II (RXR, HNF4) and subfamily III nuclear receptors (ER, GR, AR, PR) were measured in hepatocytes. In addition we hypothesized that Lcarnitine stimulates nuclear receptor expression associated with lipid catabolism (PPARα and PPARδ) accompanied with a suppression of adipogenic nuclear receptors (PPARγ) [13]. Finally, key genes of these pathways were further examined by qPCR, namely *CYP27A1, CYP2R1, ALDH1A1, HSD11B2, OGT* and *HMGCR.*

Materials And Methods

Materials

The human epithelial liver cell line WRL68 and the murine preadipocyte cell line 3T3L1 were obtained from the American Type Culture Collection (ATCC). Primary skeletal muscle cells (SKMC) were acquired from Lonza. Cignal^{TM} Finder Nuclear Receptors 10Pathway Reporter Arrays were obtained from Qiagen. All oligonucleotides for qPCR experiments were synthesized and purchased from Microsynth Austria.

Cell culture

All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

WRL68 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l Gibco^{TM} GlutaMax^{TM} (Sigma Aldrich).

SKMC cells were grown in skeletal muscle cell growth Medium (ProCell®), supplemented with 5% FCS, 50 µg/ml bovine fetuin, 10 ng/ml human epidermal growth factor (huEGF), 1 ng/ml human basic fibroblast growth factor (huFGF), 10 µg/ml recombinant human insulin and 0,4 µg/ml dexamethasone.

3T3L1 cells were maintained in DMEM supplemented with 10% FCS, 2 mmol/l Gibco™ GlutaMax™, 100 U/ml penicillin and 100 µg/ml streptomycin. To initiate adipogenesis, 3T3L1 cells were allowed to reach confluence and incubated with differentiation medium containing 1.0 µM dexamethasone, 1.0 g/ml insulin and 0.5 mM 3isobutyl1methylxanthine (IBMX; SigmaAldrich). After 48 hours of exposure to the differentiation medium, cells were maintained for an additional 7 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1.0 µg/mL Insulin. Successful adipocyte differentiation was evidenced via observation of lipid droplet formation under the microscope.

Induction of Lcarnitine deficiency and supplementation

For reporter gene assays and quantitative RealTime PCR, Lcarnitine depleted growth medium was prepared via dialysis of FCS. Dialysis was performed against 1xPBS for 72 hours with five buffer changes.

SKMC and 3T3L1 cells were kept for 48 hours in DMEM containing 10%FCS or dialyzed 10%FCS. SKMC were maintained for 48 hours in DMEM containing 5% FCS or dialyzed 5%FCS. In all cases, the cell culture medium was exchanged after 24 hours.

For reporter gene assays, Lcarnitine (Lonza) was added to the dialyzed culture medium 4 hours before harvesting either in a concentration of 40µM or 80µM to examine shorttime supplementation. In order to study extended Lcarnitine supplementation, concentrations of 40µM, 80µM and 120µM were used for 24 hours.

For qPCR experiments, additional time periods of supplementation were chosen in order to study alterations of gene expression in more detail. In order to study short-time effects, Lcarnitine was added to the dialyzed culture medium 4 hours before harvesting either in a concentration of 40µM or 80µM. Additionally, a concentration of 80µM was used 15 hours before harvesting. In order to study extended Lcarnitine supplementation, concentrations of 80µM were used for 24 and 48 hours.

Transfection and reporter gene assays

WRL68 cells were transfected using a modified version of the Lipofectamine™ 3000 Reagent Protocol (Thermo Fisher Scientific) and a Cignal Finder™ 10Pathway Reporter Array (Qiagen). 50 µl of Gibco™OptiMEM™ I Reduced Serum Medium (Thermo Fisher Scientific) were added to each well of a Cignal™ Finder Nuclear Receptors 10Pathway Reporter Array and incubated for 5 minutes at room temperature. Afterwards, 50µl OptiMEM, 0.3µl Lipofectamine™ 3000 Reagent and 0.4µl P3000™

Reagent (Thermo Fisher Scientific) were added to each well and incubated for 20 minutes at room temperature. WRL68 cells were resuspended in 7600µl OptiMEM and 400µl 10%FCS or dialyzed 10%FCS and counted with the help of a CASY cell counter. According to the number of cells, the volume for the media containing $6x10^5$ cells was calculated. 50µl of this solution were added to each well and incubated for 24 hours at 37°C, 5% CO2. The next day, the medium in the wells was completely removed and 150µl of the respective transfection medium were added to each well and again incubated for 4 or 24 hours at 37°C, 5% CO2.

Luciferase activity was determined by using the DualLuciferase® Reporter Assay System (Promega).

Reverse transcription and qPCR experiments

Total RNA was extracted from cells using the RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific).

One µg of total RNA was converted to cDNA using the LunaScript™ Reverse Transcriptase SuperMix (New England Biolabs).

Table 1

List of oligonucleotides used for this study: human qPCR primers (upper panel), murine qPCR primers (lower panel).

	forward (5'-3')	reverse (5'-3')	product size (bp)
human *β-Actin*	TCT ACA ATG AGC TGC GTG TGG	ACA GCC TGG ATA GCA ACG TAC	147 bp
human *PPAR-γ*	GCC GTG GCC GCA GAT TT	GCA GGC TCC ACT TTG ATT GC	337 bp
human *PPAR-δ*	AAG GCC TTC TCC AAG CAC AT	CAC TGG CAG CGG TAG AAG AC	227 bp
human *PPAR-α*	CAG CTG GTG CAG ATC ATC AA	CTC CCC CGT CTC CTT TGT AG	154 bp
human *RXR-α*	GGA TCC CAC ACT TCT CAG	GAG TCA GGG TTA AAG AGG AC	286 bp
human *RAR-β*	ATG GAT GTT CTG TCA GTG AG	CAT AGT GGT ACC CTGATGAT	268 bp
human *LXR-α*	CTG TCA GAA GAA CAG ATC CGC C	AAC TAT CTC CTG CAC AGA GAC GAT G	303 bp
human *OGT*	TTT GAA AGC AAT TGA GAC GCA AC	GTG CAC CAC TGC GTG ATT TG	247 bp
human *ALDH1A1*	AGA GGC AGG GTT TCC TCC TG	TTG TCC AAG TCG GCA TCA GC	228 bp
human *CYP27A1*	ACA AGG CCA AGT ACG GTC CA	TCC CGG TGC TCC TTC CAT AG	151 bp
human *CYP2R1*	GAG CGA TTT CTG GAC AGCAG	TCA GCG TCT TTC AGC ACA GA	240 bp
human *HSD11B2*	AGA AAC GTG GGT CAG TGG GA	TAC AAC TGG GGT GAG GTC GG	156 bp
human *HMG-CoA*	GGT TCG GTG GCC TCT AGT GA	GGA CAT CAT GCA GAT GGT CAG T	151 bp
murine *β-Actin*	GCG TGA CAT CAA AGA GAAG	AGG AGC CAG AGC AGT AATC	340 bp
murine *PPAR-γ*	TGG TTG ACA CAG AGA TGCCA	AGC AAC CAT TGG GTC AGC TC	182 bp
murine *PPAR-δ*	AGT CCA GCC ACA ACG CAC	AAG AAC TCG TGG GTG ACG AAG	340 bp
murine *PPAR-α*	AAA TGG TGG ACC TTC GGC AG	ATC ACC CCC ATT TCG GTA GC	324 bp
murine *RXR-α*	GAC CAG GTC ATC CTG CTA CG	GCT GCT CAG GGT ACT TGT GT	349 bp
murine *RAR-β*	CCA TAC TGG TGT CTC CAC AGC	CAT CCA ATG ACC TGG CTT CCT	234 bp
murine *LXR-α*	GAG TTA GTC TGG TGG GGA AGC	GTC TTC CAC AAC TCC GTT GC	293 bp

qPCR experiments were performed using specific primers previously checked for their ability to amplify (Table 1). qPCR was performed with a CFX96 Touch™ RealTime PCR Detection System (BioRad) using the following settings: 95°C for 30 sec, 40 cycles of 95°C for 10 sec, 60°C for 15 sec, 72°C for 40 sec. Amplification of target genes was detected via the *iTaq*™ universal SYBR® Green supermix (BioRad). βActin was used as an endogenous unregulated reference gene for all the conditions. A 5point standard curve was set up using diluted cDNA isolated from the respective cell lines. Analysis of expression levels was performed using the $^{\Delta\Delta}$CT method.

Statistical analysis

All measurements in this study were performed in triplicates and are expressed as a mean of at least two independent experiments. Mean and standard error of the mean (SEM) were calculated for each of the results.

The Student's paired ttest was used to compare mean values at each time point, obtained from cells under Lcarnitine deficiency and supplementation, with the mean value of untreated cells, which were used as a baseline control. Results with a pvalue <0.05 were considered statistically significant.

Results

Lcarnitine directly effects protein activity of nuclear receptor subfamily I members

Our first experiments examined the regulatory effect of Lcarnitine on nuclear receptor protein activity in WRL68 cells via reporter gene assay. The relative firefly luciferase activity of all subfamily I nuclear receptor pathways was increased after cultivation of cells with dialyzed FCS (Fig.1). In the case of the retinoid acid receptor pathway (RAR), shortterm induction with Lcarnitine (for 4 h) led to a further induction of the activity, whereas extended Lcarnitine supplementation for 24 hours slightly decreased activity again to 1.29fold of normal untreated cells (Fig. 1A). A similar effect was observed for the PPAR pathway (Fig. 1B). In this case, short term Lcarnitine induction even led to a 2.85fold induction of the luciferase activity.

The pathway, which was affected the most by Lcarnitine depletion and supplementation was the VitaminDreceptor pathway (Fig. 1C). Whilst the supplementation of Lcarnitine for 4 hours led to 2.88fold increase in activity, supplementing for 24 hours resulted in a reduction significantly below the normal physiological level. Contrary, in the case of the liverxreceptor pathway (LXR) the activity was further upregulated when Lcarnitine was supplemented for 24 hours (Fig. 1D).

Lcarnitine directly effects protein activity of nuclear receptor subfamily II members

Subfamily II nuclear receptor pathways were as well upregulated after induction of Lcarnitine deficiency via dialysis in WRL68 cells (Fig. 2). After shortterm Lcarnitine supplementation, the retinoid X receptor (RXR) activity was further increased 1.93fold compared to untreated cells (Fig. 2A). Extended supplementation for 24 hours led to a subsequent decrease of luciferase activity to normal physiological levels.

In the case of hepatocyte nuclear factor 4 (HNF4), a similar pattern was observed (Fig. 2B). Lcarnitine depletion and subsequent supplementation for 4 hours led to an increase in the activity of the pathway, whereas extended supplementation led to a decrease to physiological activity with rising Lcarnitine levels.

Lcarnitine only effects estrogen receptor pathway, but no other subfamily III nuclear receptors

In WRL68 cells under Lcarnitine deficiency, a 2.12fold increase of the estrogen receptor (ER) activity was observed (Fig. 3A). Addition of Lcarnitine for 4 hours led to a further upregulation of the activity. When cells were subsequently induced with 80µM or 120µM Lcarnitine for 24 hours, representing supraphysiological conditions, activity slightly decreased again.

Contrasting, androgen receptor (AR), glucocorticoid receptor (GR) and progesterone receptor (PR) activity were not significantly changed either under conditions of Lcarnitine deficiency or supplementation (Figure 3BD).

Extended Lcarnitine supplementation induces transcription of catabolic PPAR’s

To gain more insight into the regulatory role of Lcarnitine in different tissues, qPCR measurements for WRL68 cells, SKMC and 3T3L1 cells were performed. Steady state mRNA levels under Lcarnitine deficiency and supplementation were measured for the PPAR system (*α, δ, γ), RARβ, LXRα* and the obligate heterodimerization partner *RXRα.* In order to assess the time-dependent effects of Lcarnitine more precisely, additional time periods of supplementation were included compared to the reporter gene assays.

mRNA levels of *PPARα* and *PPARδ,* which are key regulators of lipid catabolism, were induced after dialysis in WRL68 and downregulated in SKMC and 3T3L1 cells (Fig. 4). *PPARγ* levels remained unchanged after dialysis in WRL68 and SKMC but were decreased in 3T3L1 cells.

Surprisingly, shorttime supplementation (for 4 hours) led to a downregulation of all PPAR members in all observed cell lines. Subsequent extended Lcarnitine supplementation led to a significant upregulation of the catabolic nuclear receptor *PPARα* in all the observed cell lines. In WRL68 cells, *PPARα* expression even was induced 7.68fold compared to normal cells. *PPARδ* mRNA amounts remained relatively decreased after prolonged supplementation in SKMC and 3T3-L1 cells but surpassed physiological levels 6.6-fold in WRL68 cells. In contrast, longterm supplementation led to a downregulation of the anabolic *PPARγ* in SKMC and a return to normal steady state mRNA levels in 3T3L1. Surprisingly, in WRL68 cells extended Lcarnitine supplementation even led to an increase of *PPARγ* mRNA amounts.

Lcarnitine supplementation significantly decreases transcription of RXR-α, *RARβ, LXRα* in SKMC and WRL68

mRNA levels of *RXRα* were downregulated after dialysis in all observed cell lines. Short-term Lcarnitine supplementation could not restore *RXR-α* expression to physiological amounts in any investigated cell line (Fig. 5A). Under extended supplementation conditions, expression levels only increased in 3T3-L1 cells.

mRNA amounts of *LXRα* and *RARβ* were as well downregulated after both dialysis and short-term supplementation in all cell lines (Fig. 5B and 5C). Surprisingly, extended Lcarnitine supplementation led to a 2fold increase of expression levels only in 3T3-L1, whilst expression levels remained significantly lowered in SKMC and WRL68 cells.

Analysis of the effect of Lcarnitine on the expression levels of key genes of nuclear receptor pathways

Based on the results of the examined nuclear receptor pathways, we further examined possible changes in transcription levels of key effector genes in WRL68 cells, since Lcarnitine is mainly metabolized in liver tissue in humans. Cytochrome P450 Family 27 Subfamily A Member 1 (*CYP27A1)* expression remained unchanged after dialysis and was decreased after shorttime Lcarnitine supplementation (Fig. 6A). Subsequent supplementation for 48 hours led to a return of the steady state mRNA levels to normal heights of expression. Cytochrome P450 Family 2 Subfamily R Member 1 (*CYP2R1)* gene expression was increased significantly in cells grown in dialyzed FCS (Fig. 6B) and remained slightly increased after shorttime supplementation. However, there was a 5.22fold increase in *CYP2R1* gene expression, when cells were supplemented with 80 µM Lcarnitine for 24 hours and an even higher 50.83fold upregulation at the 48 hourstimepoint.

Alcohol dehydrogenase 1A1 (*ALDH1A1)* expression was decreased significantly after induction of Lcarnitine deficiency (Fig. 6C). Both shortterm Lcarnitine supplementation as well as extended Lcarnitine supplementation could not upregulate transcription, and the expression levels remained at 15% compared to normal untreated cells.

11βhydroxysteroid dehydrogenase type 2 (*HSD11B2)* expression rose slightly 1.42fold when cells were grown under Lcarnitine deficiency condition (Fig. 6D). Lcarnitine supplementation for 4 hours, 15 hours and 24 hours only led to a slight increase of expression. However, when supplementing for 48 hours, gene expression was dramatically increased 34.72fold.

Olinked βNacetylglucosamine transferase (*OGT)* expression remained unchanged after dialysis (Fig. 6E). Whilst shortterm Lcarnitine supplementation only slightly altered expression levels, the expression was decreased 0.73-fold compared to the control group after 48 hours of supplementation.

3hydroxy3methylglutarylCoA (HMGCoA) reductase (*HMGCR)* expression was increased after depletion of Lcarnitine and remained on an elevated level after shortterm supplementation (Fig. 6F). The effect was further amplified at the 48 hourstimepoint, where the gene expression increased 7.18fold.

Discussion

The current study is the first to demonstrate the distinct effects of Lcarnitine deficiency and supplementation on nuclear receptors in general and on PPARs in adipocytes, liver- and muscle cells in a comparative manner. No detailed studies have been published so far comparing the effects of short-term and extended Lcarnitine substitution on the expression levels and activity of nuclear receptors. Some results regarding the effects of Lcarnitine on the nuclear level have been found already, however all papers only described effects over one time period of supplementation. *In vitro* substitution of Lcarnitine for 24 hours in 3T3L1 cells led to a upregulation of lipolytic genes, like hormonesensitive lipase, as well as a significant downregulation of *PPARγ [*9]. Since PPARγ was proven to be involved in adipogenesis, this would suggest a role of Lcarnitine as a stimulator of lipolysis and energy dissipation. A corresponding effect was found in the case of *PPARα* in rats, where in vivo substitution of Lcarnitine for 28 days led to a significant upregulation of *PPARα* expression [14]. PPARα is already known to play a crucial role as an enhancer of fatty acid oxidation, therefore substantiating the role of Lcarnitine as an important stimulator of the catabolism.

By reportergene assays, we were able to prove a direct effect of Lcarnitine on nuclear receptor protein activity (Fig. 1–3). The protocol for induction of Lcarnitine deficiency with the use of dialyzed FCS is an established procedure and has been previously used in publications from our research group [8, 10]. Lcarnitine deficiency, achieved via dialysis, in WRL68 cells led to an initial increase of seven examined nuclear receptor pathways (RAR, RXR, VDR, PPAR, HNF4, ER, LXR). No changes could be observed in the AR, PR and GR pathway, where the relative firefly luciferase activity remained at the level of physiological cultivation conditions. Subsequent shortterm Lcarnitine supplementation for 4 hours resulted in a further stimulation of the seven above mentioned pathways. Short term Lcarnitine pulses for 4 hours can only address preformed transcription complexes, because no significant de novo protein synthesis can take place in this limited time period. Therefore, Lcarnitine substitution obviously has the potential to interact with preformed nuclear receptor complexes, thereby increasing pathway signaling activity.

The pathways which were affected the most by 4 hours of Lcarnitine supplementation were the VDR and PPAR pathway. Interestingly, a link between these two pathways has already been revealed, with 1,25dihydroxyvitamin D being able to upregulate the PPAR pathway and thereby stimulating lipid metabolism in diabetic rats [15]. The PPAR signaling pathway is already known to be influenced by numerous metabolites, thereby acting as a key regulator of lipid homeostasis [16, 17]. Since Lcarnitine serves as an essential esterefication partner of fatty acids getting transported across the mitochondrial membrane, alterations in intracellular Lcarnitine levels have a direct consequence for the lipid metabolism. Based on our results, Lcarnitine levels have a direct effect on the PPAR activity, thereby adjusting lipid catabolism depending on how much Lcarnitine is available to import lipids into the mitochondria.

Lcarnitine supplementation for 24 hours only led to an increase of activity in the case of the LXR and ER pathway. Regarding the nuclear receptor vitamin D pathway, a 24 hours lasting Lcarnitine supplementation exerted a diverging timedependent effect. The respective pathway activity was upregulated after 4 hours, but suppressed significantly after 24 hours of L-carnitine supplementation. The activity of the RAR, RXR, HNF4 and PPAR pathway were downregulated to physiologial steady state mRNA levels after 24 hours of supplementation.

In addition to this effect, we were able to reveal a further close connection between the PPARsystem and Lcarnitine on the transcriptional level in different tissues. Therefore, primary human skeletal muscle cells were investigated together with the WRL68 cell line, representative of mature human hepatocytes, as well as differentiated 3T3L1 cells, representative of mature murine adipocytes. The combination of human hepatocyte cell lines and murine adipocyte cell lines has already been successfully used in previous studies [18, 19].

PPARγ, PPARα and *PPARδ* steady state mRNA levels in WRL68 cells were upregulated in the absence of Lcarnitine via the use of dialyzed FCS, according to the results of the reporter gene assays (Fig. 4). In contrast, in SKMC and 3T3L1 cells, dialysis led to a downregulation of the whole PPAR system. In addition, transcript amounts of all PPAR members were downregulated in all three observed cell lines in the course of shortterm Lcarnitine supplementation for 4 hours, whilst the reporter gene assay showed an increase of PPAR activity after short-term supplementation. This observation further substantiates the hypothesis that Lcarnitine is able to rapidly interact with preformed protein complexes on the nuclear level.

In contrast to the short time pulse, extended Lcarnitine supplementation for 24 and 48 hours led to a slight increase of *PPARγ* transcription levels in WRL68 cells. *PPARδ* and *PPAR-α* mRNA amounts in WRL68 were markedly increased 6.7- and 7.7-fold, respectively. In contrast, in the reporter gene assay for PPAR protein signaling, activity decreased down to physiological levels in the course of 24 hours of Lcarnitine supplementation. A possible reason for this disparity could be the transcriptional downregulation, which was observed after 4 hours and 15 hours of supplementation. Steady state mRNA levels were significantly downregulated after short-term supplementation and did not increase again up

until 24 hours of supplementation. The most pronounced upregulation of transcription was observed in the case of 48 hours of supplementation. Thus, possible effects of extended Lcarnitine supplementation on nuclear pathway protein activity could probably be observed via protein activity measurements after more than 24 hours. Therefore, effects of L-carnitine supplementation for longer time periods of supplementation on the protein activity of nuclear receptors would be worthwhile being investigated in future studies.

Whereas, extended supplementation in 3T3L1 cells led to a return of the *PPARγ* steady state mRNA levels to normal degrees and to a downregulation of *PPARγ* in SKMC. An upregulation of *PPAR-γ* was only observed in the case of WRL68. These divergent effects can be directly explained by the different functions of PPAR-γ in the investigated cell lines, as described by Dubois et al. [13]. Few studies on the effects of Lcarnitine supplementation in skeletal muscles have been published so far. Interestingly, Lcarnitine substitution for 6 weeks in rats led to an increased expression of *PPARγ* in skeletal muscles [20]. Since our experiments only measured the expression levels after supplementation for 4 hours to 48 hours, Lcarnitine supplementation over the course of weeks may lead to a different expression pattern as part of a response to changing metabolic conditions. Thus, possible effects of Lcarnitine supplementation on nuclear receptor expression for longer time periods are worthwhile being investigated in the future.

Different to *PPARγ,* the transcript levels of *PPARα* were significantly increased in all the cell lines after extended L-carnitine supplementation, concordant with previous studies [9]. *PPAR-δ* mRNA amounts remained relatively lowered in SKMC and 3T3-L1 cells after prolonged L-carnitine supplementation but were as well significantly increased 6.7-fold in WRL68 cells. PPARγ upregulation is known to lead to an increase of lipogenesis and a decrease of lipolysis in adipose tissue [21]. In contrast, PPARα and PPARδ promote an increase of βoxidation in skeletal muscles and hepatocytes as well as a decrease of de novo lipogenesis in the liver [13]. Therefore, based on our results, extended Lcarnitine supplementation seems to be able to potently promote catabolic pathways, by inducing expression of catabolic PPARs and thus enhancing βoxidation and reducing lipogenesis.

The additional promoter active factors *LXRα* and *RARβ* were downregulated following the absence of Lcarnitine in all cell lines. In SKMC and WRL68 cells, mRNA levels remained significantly lowered after both short-term and extended supplementation. In WRL68 cells, steady state expression levels even dropped down to 2% under dialysis and remained at 15% even after extended supplementation, compared to normal growth conditions. These results show a clear disparity between decreasing mRNA abundance and the increase of LXR-α and RAR-β protein activity in WRL68 cells observed in the reporter gene assays (Fig. 1–2). This phenomenon could be due to several reasons. Whilst transcription is downregulated, the pool of already synthesized mRNA might however be more efficiently translated. Another reason could be an increase of the proteins' halflife due to a reduced rate of degradation via posttranslational modifications. Lcarnitine administration has already been shown to modulate posttranslational modifications, leading to an increase of phosphorylation of AMPactivated protein kinase (AMPK), PI3K, Akt and mTOR [22–24]. Additionally, Lcarnitine supplementation has been proven to exert a decrease in

proteasome activity and downregulate genes of the ubiquitin proteasome system [25, 26]. A similar effect has been described for the nutrigenomic metabolite Niacin, where supplementation reduced ubiquitination of hepatic ACOX1 and CYP4A1, thereby increasing protein levels without affecting mRNA transcription [27].

Interestingly, 3T3-L1 was the only cell line, where an increase of *LXR-α* and *RAR-β* transcription was observed after extended L-carnitine supplementation (Fig. 5). After 48 hours of supplementation, mRNA levels increased up to 2-fold compared to the normal untreated cells. LXR activation in both murine and human adipocytes has already been shown to shift substrate oxidation towards utilization of lipids and upregulate mitochondrial β-oxidation [28]. Concordant with these findings, RAR activation in adipocytes seems to suppress adipogenesis and to down-regulate mRNA expression of *PPAR-γ,* a key regulator of adipocyte differentiation [29, 30]. These results further substantiate our hypothesis, that L-carnitine acts as potent promoter of catabolic pathways via increase of lipolysis and decrease of lipid storage in multiple tissues.

Interestingly, the expression levels of *RXR-α* increased as well only in 3T3-L1 cells after extended supplementation. In contrast, in SKMC and WRL68 cells, *RXR-α* mRNA amounts declined under Lcarnitine deficiency but remained relatively unchanged in all states of supplementation, averaging at two-thirds of the expression levels observed in normal growth conditions. A possible reason for this could be the fact that RXRα acts as an obligate hetero-dimerization partner for a number of other nuclear receptors [31]. Since a strong decrease in RXRα levels would also impair the effectiveness of those other receptors, it could be hypothesized that cells must maintain relatively high amounts of RXRα in order to preserve their capacity to modulate transcription of essential target genes.

In addition, the effect of Lcarnitine on the expression levels of key effector genes in WRL68 cells was measured (Fig. 6). *ALDH1A1* is a major gene of the oxidative pathway of alcohol metabolism and has been shown to act as a promoter of adipogenesis [29, 30]. Lcarnitine supplementation led to a significant decrease of *ALDH1A1* expression, substantiating the hypothesis that Lcarnitine substitution effectively decreases adipogenesis. Olinked βNacetylglucosamine transferase (OGT) catalyzes the addition of Nacetylglucosamine in Oglycosidic linkage to serine or threonine residues [32]. Additionally, hepatic OGT overexpression has been shown to impair the expression of insulinresponsive genes and contribute to insulin resistance and dyslipidemia [33]. Extended Lcarnitine supplementation for 48 hours led to a 0.7-fold reduction of *OGT* expression, which further elucidates

the beneficial role of Lcarnitine on metabolic pathways. Additionally, extended Lcarnitine supplementation led to a strong increase of key genes of the VDR pathway *CYP27A1* and *CYP2R1.* In the case of *CYP2R1,* supplementation for 48 hours even led to a dramatic 50fold upregulation of mRNA transcription. *HMGCR,* a key gene of the LXR pathway, and *HSD11B2,* a key gene of the GR pathway, were as well upregulated 7.2- and noteworthy 35-fold, respectively. These findings provide additional support for our observations, which indicate that Lcarnitine directly modulates both nuclear receptor gene transcription as well as transcription of key effector genes. In our current study, changes in expression

levels of effector genes were measured in WRL68 hepatocytes, because physiologically Lcarnitine is mainly metabolized in liver tissue. In order to assess if the expression of those key genes diverges as well between different tissues after L-carnitine supplementation, changes of effector gene expression levels in other cell lines would be as well worthwhile being investigated in future studies.

Beyond that, in a chipscreen study performed by our lab, we observed that several hundreds of genes throughout the whole genome showed an increased or decreased transcription due to Lcarnitine supplementation, underlining the importance of this nutrigenomic metabolite [54]. Similar intense effects on the transcriptome, which resulted in altered expression of hundreds of genes, were already observed for the nutrigenomic metabolites niacin, vitamin D and leucine [55–57]. In the case of niacin, similar to our results, a tissuespecific pattern of effects has been observed as well. Niacin specifically altered expression of a group of genes in adipose tissue, but not in the liver, heart or skeletal muscle [57]. For a more detailed representation, Table 2 shows a comparison of Lcarnitine with other important nutrigenomic metabolites exerting similar effects.

Table 2

Comparison of nutrigenomic metabolites, determined by observed metabolic effects and examples of affected genes

Nutrient	Effects	Affected genes	References
Leucine	Increase of glucose uptake, lipid storage and protein synthesis	nuclear receptors: *PPAR-δ* key genes*: GLUT4, PK, GST*	[34–36]
Niacin (vitamin B3)	Significant tissue-dependent effects. Modulation of specific protein activity without alteration of mRNA transcription Positive effects on cardiovascular events and atherogenesis	nuclear receptors: *PPAR-α, LXR* key genes: *L-FABP, CD36, OCTN2, CYP4A1, ACOX1*	[27, 37–39]
Vitamin D	Positive effects on insulin resistance and glucose uptake Prevention of oxidative stress	nuclear receptors: *VDR, PPAR-γ* key genes: *SIRT1, GLUT4, NOX4*	[40–42]
Retinol (Vitamin A)	Possibly beneficial effects on suppressing adipogenesis and atherogenesis	nuclear receptors: *RAR-α, RAR-γ, RAR-β, NGF, RXR-α, RXR-β, RXR-γ* key genes: *IL-17, RORc, Il-10, HSP70*	[43–45]
Flavonoids	Anti-inflammatory and antioxidative properties	nuclear receptors: *ER, PR, PPAR-γ* key genes: *Nrf2, NFκB, LDH, SOD, GPx*	[46–49]
Zinc	Increase of fatty-acid oxidation. Anti-inflammatory properties and improvement of immune response	nuclear receptors: *PPAR-γ, PPAR-α, HNF4α* key genes: *CPT1A, ALDH2, TNF-α, IFN-γ*	[50–53]
L-carnitine	Modulation of expression and protein activity of nuclear receptors. Significant tissue-dependent effects Positive effects on lipolysis, decrease of adipogenesis	nuclear receptors: *PPAR-α, PPAR-γ, PPAR-δ, RXR-α, RAR-β, LXR-α* key genes: *CYP27A1, CYP2R1, ALDH1A1, HSD11B2, OGT, HMGCR*	[6, 7, 9]

Conclusion

The current study is the first to demonstrate the distinct timedependent effects of Lcarnitine on cellular pathways on multiple levels. Our approach included measurements of protein activity, nuclear receptor transcription levels as well as transcription levels of key effector genes. The results provide strong evidences that Lcarnitine has a direct effect on nuclear receptor protein activity. Furthermore, we were able to show that Lcarnitine acts as a potent inducer of the transcription of catabolic nuclear receptors and decreases transcription of adipogenic and insulinresistance linked effector genes. Additionally, diverging effects of Lcarnitine supplementation on PPAR transcription levels in different tissues could be shown in the interplay of adipocytes, skeletal muscle cells and hepatocytes. Based on our results, Lcarnitine supplementation might be a promising candidate in supporting established antihyperlipidemic therapy, by obeying distinct rules and caveats of its clinical use [58]. Therefore, future studies on the complex effects of Lcarnitine on cellular pathways are necessary to extend our understanding of the key molecular mechanisms behind its apparent beneficial effects.

Declarations

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Authors' contributions

DI performed culturing of WRL68 cells, transfection, reporter gene assays and qPCR experiments for key effector genes in WRL68 cells. LF performed culture of SKMC and 3T3-L1 cells and all other qPCR experiments. DI and LF both carried out statistical analysis and participated in drafting the manuscript and the figures. RH planned the study and supervised the experiments, participated in sampling and finalized the illustrations and the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

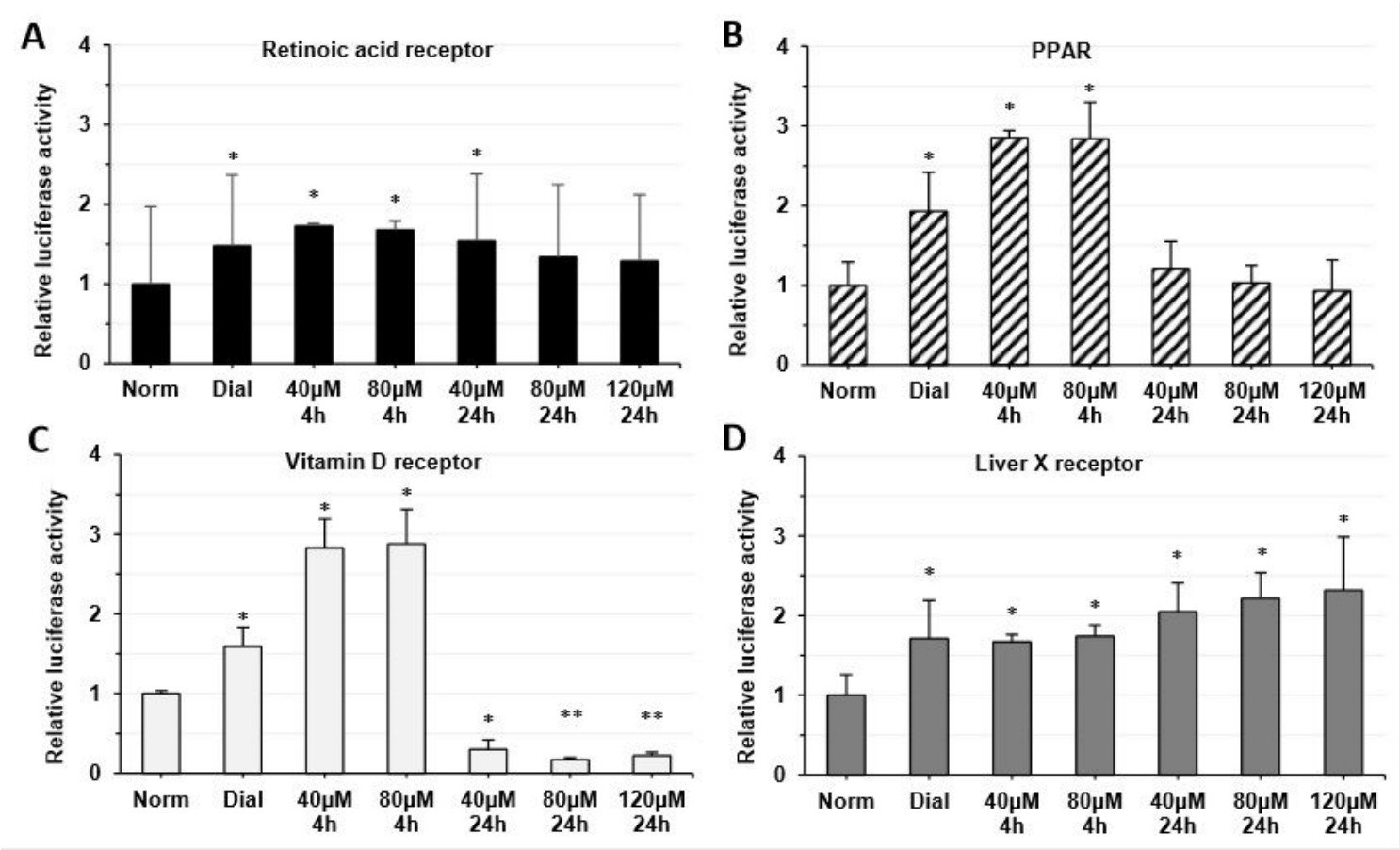

Figure 1

Relative firefly luciferase activity of Type I nuclear in WRL68 cells. (A) retinoic acid receptor pathway. (B) PPAR pathway. (C) vitamin D receptor pathway. (D) liver X receptor pathway. Values represent means ± SEM. Means without asterisk show no statistical significance ($p > 0.05$); (p-values of asterisk marked means: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

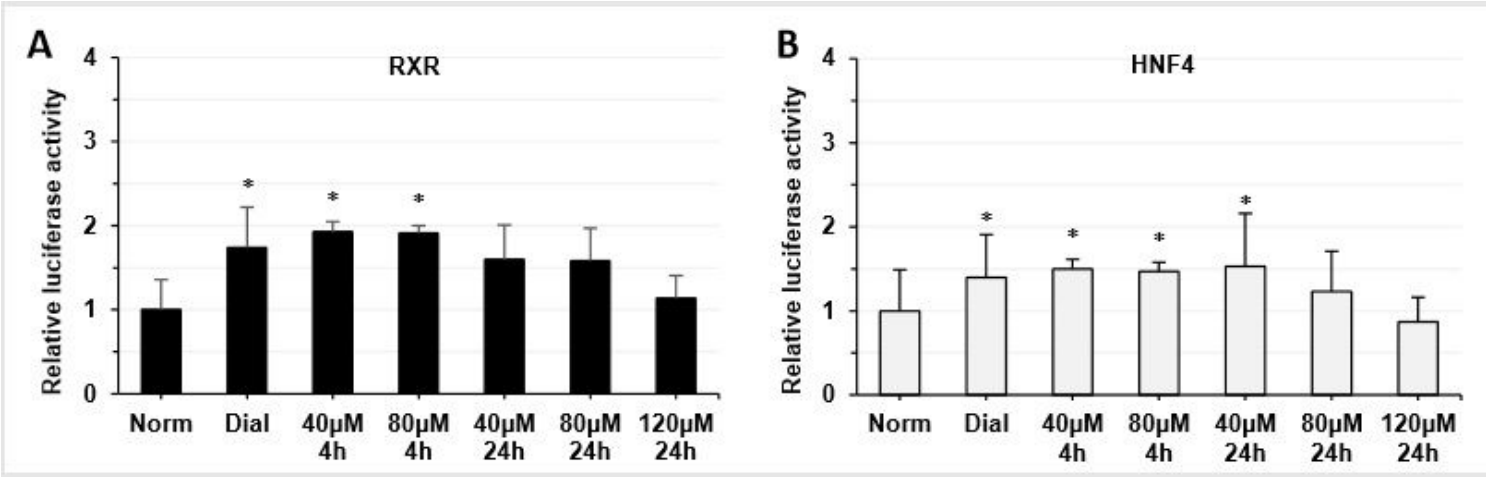

Figure 2

Relative firefly luciferase activity of Type II nuclear receptors in WRL68 cells. (A) retinoid X receptor pathway. (B) hepatocyte nuclear factor 4 pathway. Values represent means ± SEM. Means without

asterisk show no statistical significance (p > 0.05); (p-values of asterisk marked means: *p < 0.05, **p < 0.01, ***p < 0.001).

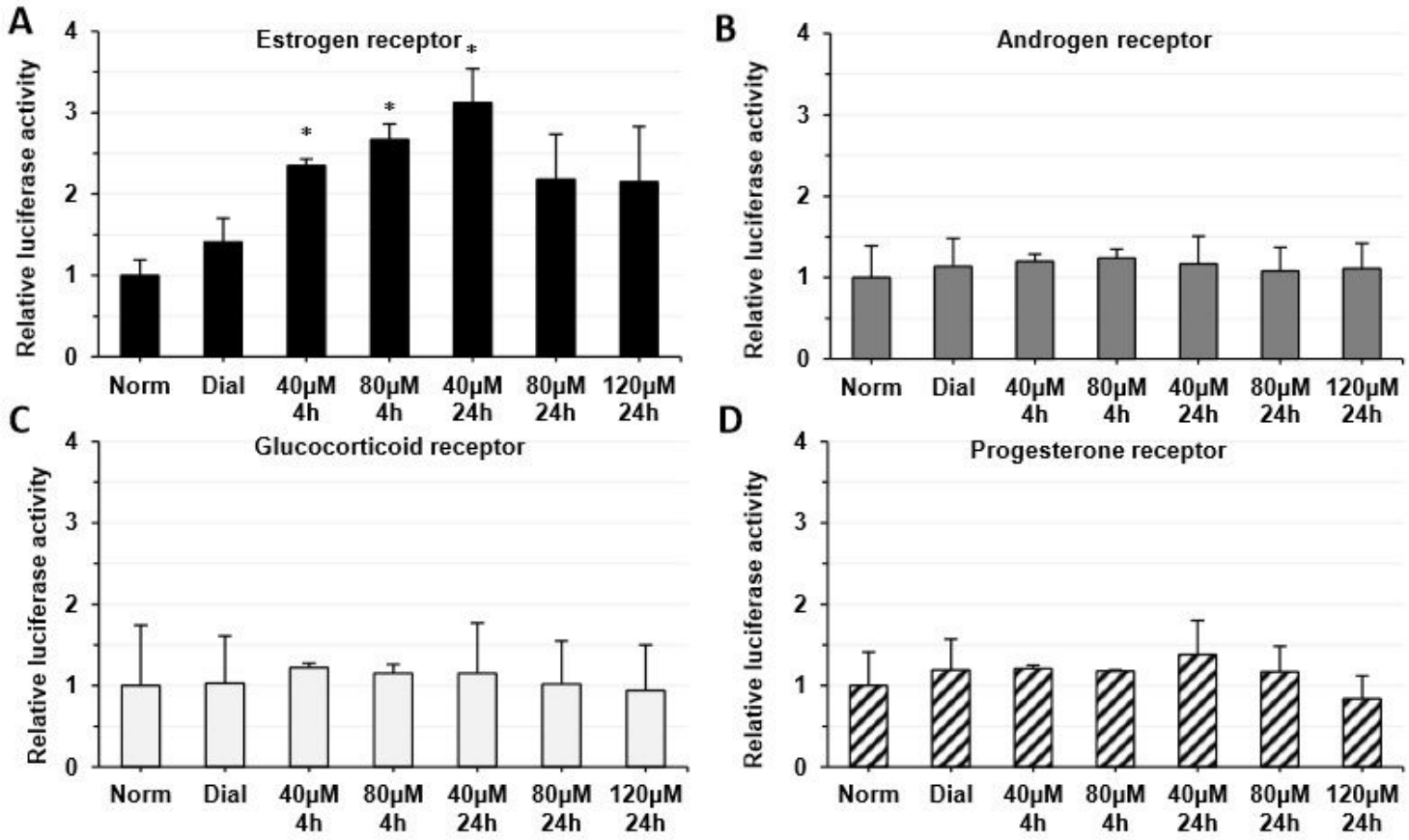

Figure 3

Relative firefly luciferase activity of Type III nuclear receptors in WRL68 cells. (A) estrogen receptor pathway. (B) androgen receptor pathway. (C) glucocorticoid receptor pathway. (D) progesterone receptor pathway. Values represent means ± SEM. Means without asterisk show no statistical significance (p > 0.05); (p-values of asterisk marked means: *p < 0.05, **p < 0.01, ***p < 0.001).

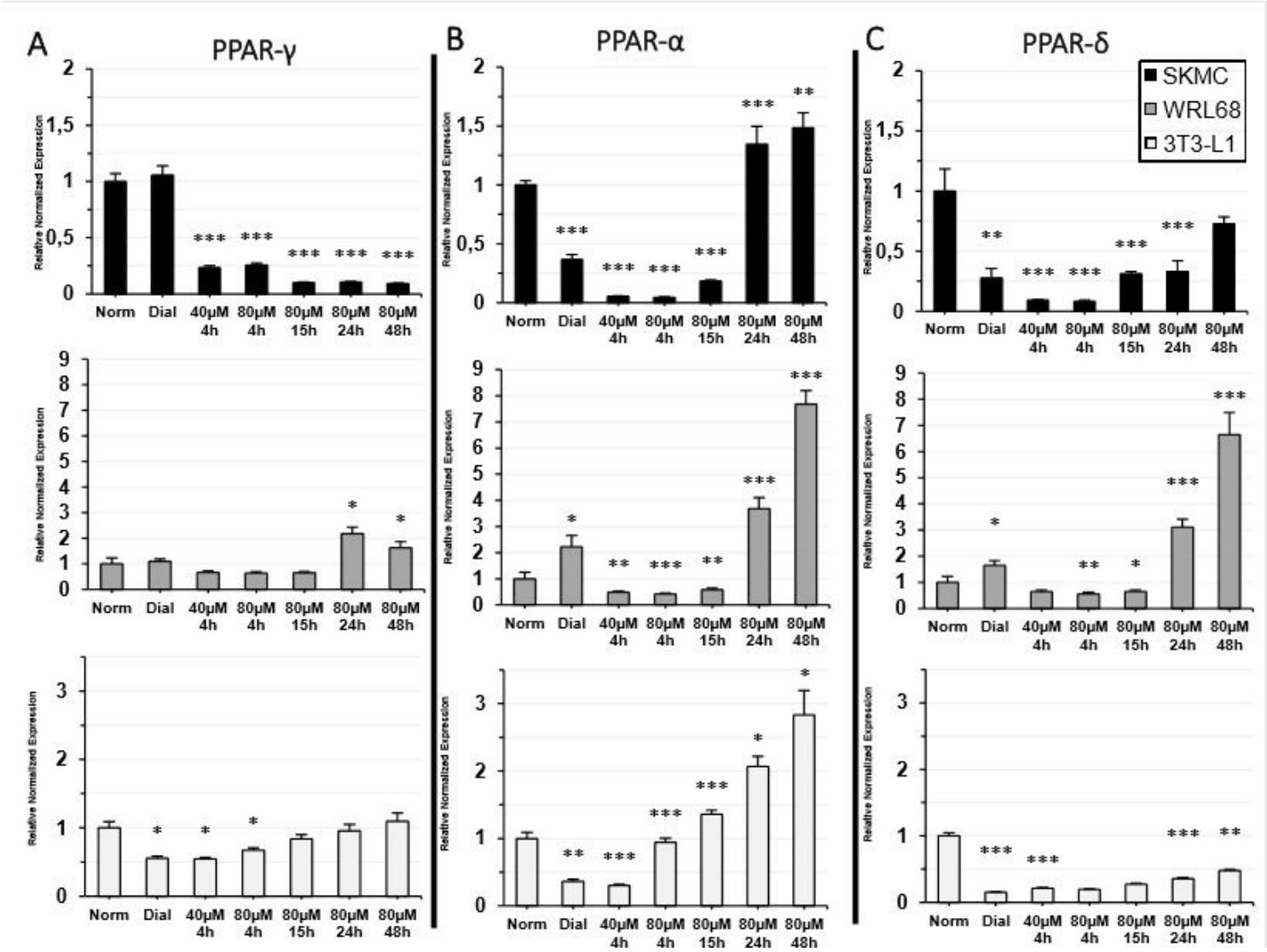

Figure 4

Expression of PPARs in SKMC, WRL68 cells and 3T3-L1. (A) PPAR-γ. (B) PPAR-α. (C) PPAR-δ. Values represent means ± SEM. Means without asterisk show no statistical significance ($p > 0.05$); (p-values of asterisk marked means: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

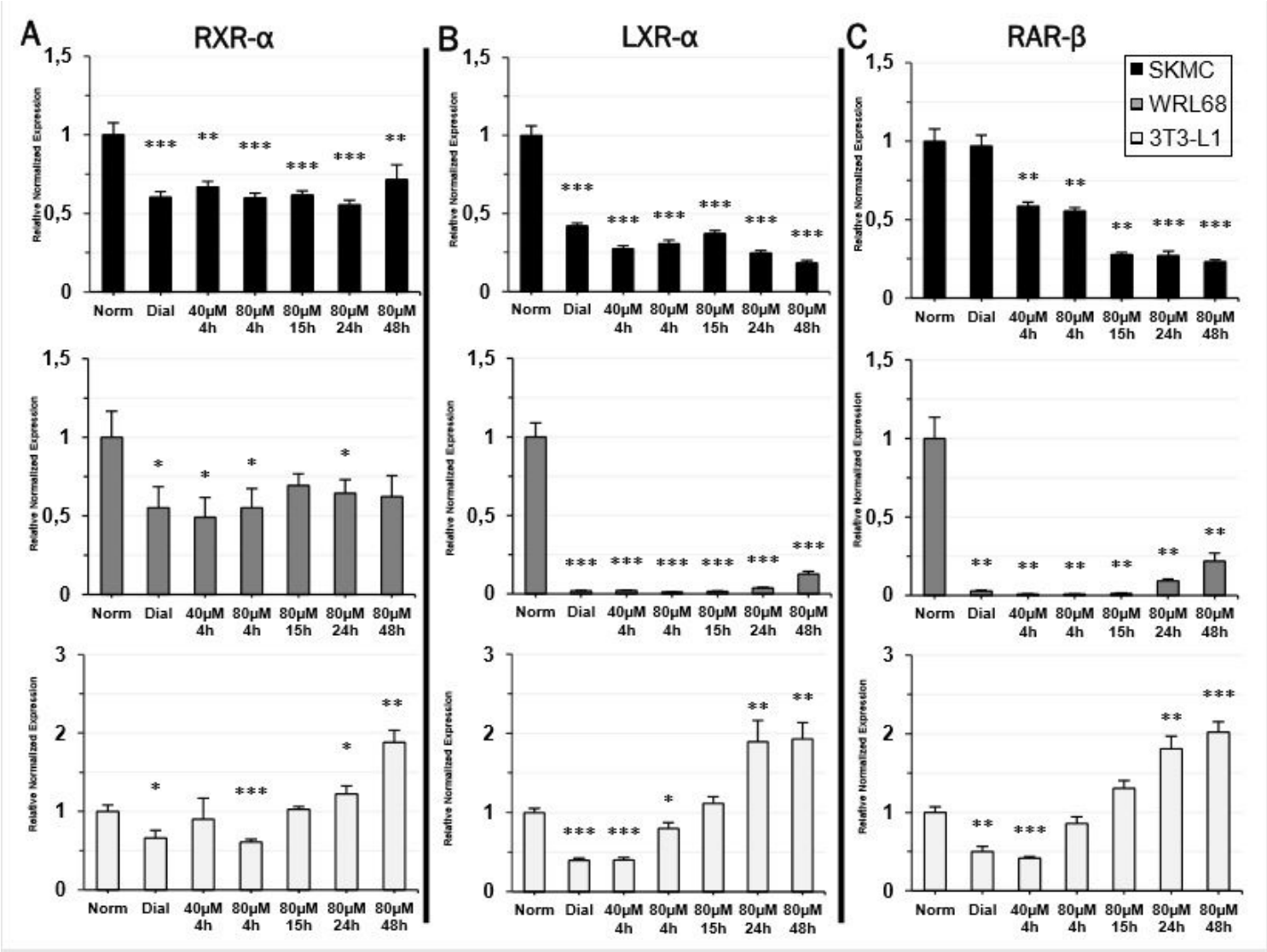

Figure 5

Expression of additional nuclear receptors in SKMC, WRL68 cells and 3T3-L1. (A) RXR-α. (B) LXR-α. (C) RAR-β. Values represent means ± SEM. Means without asterisk show no statistical significance ($p > 0.05$); (p-values of asterisk marked means: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

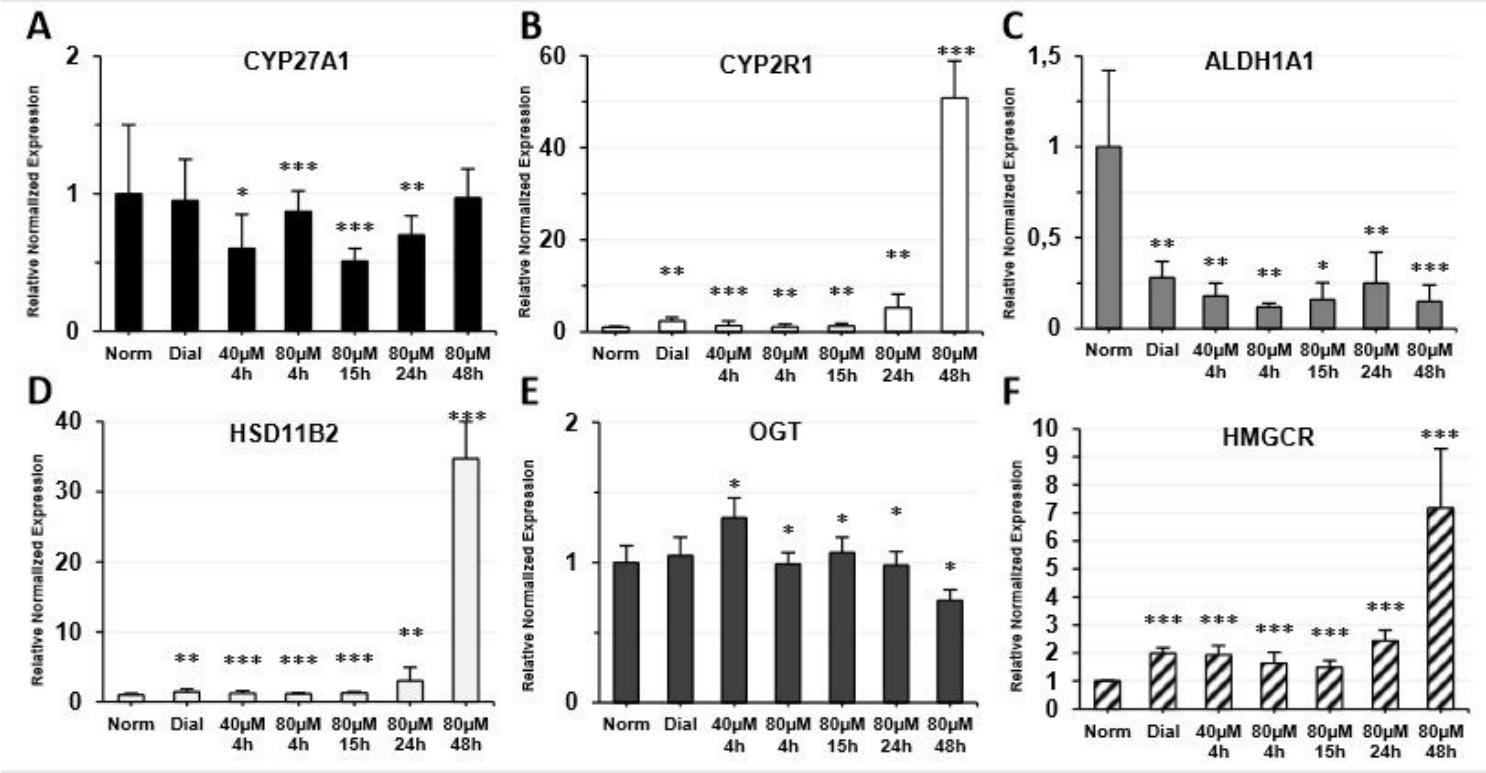

Figure 6

Expression of key effector genes under in WRL68 cells. (A) CYP27A1. (B) CYP2R1. (C) ALDH1A1. (D) HSD11B2. (E) OGT. (F) HMGCR. Values represent means ± SEM. Means without asterisk show no statistical significance ($p > 0.05$); (p-values of asterisk marked means: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).