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Conjugated linoleic acid isomers and their precursor fatty acids regulate peroxisome proliferator-activated receptor subtypes and major peroxisome proliferator responsive element-bearing target genes in HepG2 cell model*

Sailas BENJAMIN†[1,2], Silke FLOTHO[1], Torsten BÖRCHERS[1], Friedrich SPENER[1,3]

([1]*Department of Biochemistry, University of Münster, 48149 Münster, Germany*)

([2]*Enzyme Technology Laboratory, Biotechnology Division, Department of Botany, University of Calicut, Kerala 673 635, India*)

([3]*Department of Molecular Biosciences, Lipidomics Research Center, University of Graz, Heinrichstrasse 31, 8010 Graz, Austria*)

†E-mail: sailasben@yahoo.co.in

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Abstract: The purpose of this study was to examine the induction profiles (as judged by quantitative reverse transcription polymerase chain reaction (qRT-PCR)) of peroxisome proliferator-activated receptor (PPAR) α, β, γ subtypes and major PPAR-target genes bearing a functional peroxisome proliferator responsive element (PPRE) in HepG2 cell model upon feeding with *cis*-9,*trans*-11-octadecadienoic acid (9-CLA) or *trans*-10,*cis*-12-octadecadienoic acid (10-CLA) or their precursor fatty acids (FAs). HepG2 cells were treated with 100 μmol/L 9-CLA or 10-CLA or their precursor FAs, viz., oleic, linoleic, and *trans*-11-vaccenic acids against bezafibrate control to evaluate the induction/expression profiles of PPAR α, β, γ subtypes and major PPAR-target genes bearing a functional PPRE, i.e., fatty acid transporter (FAT), glucose transporter-2 (GLUT-2), liver-type FA binding protein (L-FABP), acyl CoA oxidase-1 (ACOX-1), and peroxisomal bifunctional enzyme (PBE) with reference to β-actin as house keeping gene. Of the three housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, and ubiquitin), β-actin was found to be stable. Dimethyl sulfoxide (DMSO), the common solubilizer of agonists, showed a significantly higher induction of genes analyzed. qRT-PCR profiles of CLAs and their precursor FAs clearly showed upregulation of FAT, GLUT-2, and L-FABP (~0.5–2.0-fold). Compared to 10-CLA, 9-CLA decreased the induction of the FA metabolizing gene ACOX-1 less than did PBE, while 10-CLA decreased the induction of PBE less than did ACOX-1. Both CLAs and precursor FAs upregulated PPRE-bearing genes, but with comparatively less or marginal activation of PPAR subtypes. This indicates that the binding of CLAs and their precursor FAs to PPAR subtypes results in PPAR activation, thereby induction of the target transporter genes coupled with downstream lipid metabolising genes such as ACOX-1 and PBE. To sum up, the expression profiles of these candidate genes showed that CLAs and their precursor FAs are involved in lipid signalling by modulating the PPAR α, β, or γ subtype for the indirect activation of the PPAR-target genes, which may in turn be responsible for the supposed health effects of CLA, and that care should be taken while calculating the actual fold induction values of candidate genes with reference to housekeeping gene and DMSO as they may impart false positive results.

Key words: Conjugated linoleic acid, HepG2 cell model, qRT-PCR, Peroxisome proliferator-activated receptor, Peroxisome proliferator responsive element-bearing genes, Lipid agonists

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1 Introduction

Conjugated linoleic acids (CLAs) are positional and geometric isomers of linoleic acid found predominantly in milk, milk products, meat, and meat

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products of ruminants (Benjamin and Spener, 2009). Together with *trans*-11-octadecenoic acid (*trans*-11-vaccenic acid (VA)), the CLAs are formed as the by-products of microbial biohydrogenation of oleic (OA) and linoleic acids (LA) in the rumen of the ruminants (Bauman and Griinari, 2001; Liu *et al*., 2012). A further share in producing the main CLA isomer, viz., *cis*-9,*trans*-11-octadecadienoic acid (9-CLA), is provided via food chain entry of VA into mammalian (including human) system, where it is desaturated at Δ^9 by stearoyl-CoA desaturase (Griinari *et al*., 2000). The destiny of CLA in mammalian systems is storage as neutral lipids such as OA-like molecules in fatty tissues, or metabolized as LA-like molecules by further desaturation and elongation in other organ cells, or finally subjected to peroxisomal β-oxidation (Belury *et al*., 2007; Zhao *et al*., 2008). CLAs bind to peroxisome proliferator-activated receptor (PPAR) subtypes α, β, and γ (Benjamin and Spener, 2009), which in turn heterodimerize with a subtype of retinoic acid receptor for interaction with a peroxisome proliferator responsive element (PPRE) in the promotor region of a target gene (Benjamin and Spener, 2009). This activation initiates gene transcription, and thus CLAs reveal their potential to regulate lipid metabolism and in part carbohydrate homeostasis (Moya-Camarena *et al*., 1999; Belury *et al*., 2007). Functional PPREs have been reported for many PPAR-target genes involved in intracellular lipid transport and metabolism. Among them, major representatives of membrane transporters of the lipid ligands/agonists are the fatty acid transporter (FAT) or cluster of differentiation 36 (CD36) (Sato *et al*., 2002), glucose transporter-2 (GLUT-2) (Kim *et al*., 2000), and liver type-fatty acid binding protein (L-FABP) (Schachtrup *et al*., 2004). Those involved in metabolism are acyl CoA oxidase-1 (ACOX-1) and peroxisomal bifunctional enzyme (PBE) (Kassam *et al*., 2001), which constitute the key downstream peroxisomal targets. We have previously illustrated the interplay of these genes during cell signaling (Benjamin and Spener, 2009).

In fact, 9-CLA and *trans*-10,*cis*-12-octadecadienoic acid (10-CLA) have become the primary focus for the study of many biological activities, and have health benefits (Aldai *et al*., 2008). Reportedly, by controlled consumption, CLAs exert "functional food" (Benjamin and Spener, 2009) effects in curbing atherosclerosis, cancer, diabetes, obesity, oxidative stress, immuno-modulation, and enhancing bone formation and its composition (Park and Pariza, 2007; Benjamin and Spener, 2009). The thrust for CLA-mediated health benefits mainly sprouts from animal studies supported by in vitro cell culture models, with very little information from human studies (Belury *et al*., 2007; Park and Pariza, 2007; Benjamin and Spener, 2009).

In mouse primary hepatocytes as well as human HepG2 cell lines, we previously demonstrated that L-FABP interacts with PPARα and PPARγ. As a result, a fatty acid (FA) signal transducing cascade proceeds into the nucleus, where the FA ligands for L-FABP become agonists of nuclear receptors regulating gene expression (Wolfrum *et al*., 2001). In a preceding study, we found that both 9-CLA and 10-CLA are bound by all three PPAR subtypes (dissociation constant K (K_{DS}) from 600 to 1300 nmol/L), and showed that the affinity of 9-CLA (as compared to 10-CLA) was 2-fold higher for PPARα, 3-fold lower for PPARβ, and equal for PPARγ (Benjamin *et al*., 2005). In contrast, CLA precursor acids, viz., OA, LA, and VA, were bound by PPARα and PPARγ only, with LA having the lowest affinity for both the nuclear receptors (Benjamin *et al*., 2005). Subsequent screening of all FAs mentioned above for PPAR transactivation with an optimized molecular test system revealed that ligand/agonist bound by L-FABP and PPARs, respectively, does not allow for prediction of the FA's transactivation potentials (Benjamin *et al*., 2005).

Based on our scientific rationale for studying interactions between lipid transport proteins, PPARs and PPRE-bearing target genes referred to above, we now ask the question whether or not CLAs and their precursor FAs affect interdependent expression of genes encoding PPARs and genes targeted by PPAR subtypes at transcriptional levels. This includes the logical follow-up investigation on the correlation of FA's transactivation potentials reported in the preceding paper (Benjamin *et al*., 2005) with expression of target genes determined here.

Thus, the experimental rationale in this study makes use of the human HepG2 cell culture by incubating cells with 9-CLA and 10-CLA, OA, LA, VA, and the positive control bezafibrate (BZF). Expressions of PPAR subtypes and their target genes

involved in lipid and carbohydrate homeostasis are analyzed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) under optimized conditions. Representative PPAR-target genes employed in this study were transporter L-FABP (Schachtrup *et al*., 2004), membrane-bound FA transporter FAT/CD36 (Sato *et al*., 2002) and glucose transporter GLUT-2 (Kim *et al*., 2000), and, in addition, FA-catabolising enzymes like ACOX-1 and PBE (Kassam *et al*., 2001) were also focused in this study as specific objectives.

2 Materials and methods

2.1 Administration of FAs to HepG2 cell cultures

HepG2 cell culture and feeding conditions have been described in detail by Benjamin *et al*. (2005). Briefly, when cells were about 75% confluent, fetal calf serum (FCS)-containing RPMI-1640 medium was replaced by FCS-free fortified RPMI-1640 medium, containing 100 μmol/L of respective FA ligand/agonist or the positive control BZF and FA-free bovine serum albumin (BSA) (molar ratio of FA/BSA 5:1). Earlier we optimized the concentration of FA agonists as 100 μmol/L, wherein 50, 100, and 200 μmol/L concentrations were tested (Benjamin *et al*., 2005). FAs were applied in dimethyl sulfoxide (DMSO), whose final concentration in the medium always was 1%. After 24 h incubation, cells were harvested and lysed with lysis buffer supplied by Qiagen RNeasy kit (Hilden, Germany) for extracting total RNA. 9-CLA (~90% purity) and 10-CLA (~95% purity) were purchased from Larodan fine chemicals (Malmö, Sweden), other FAs, BZF, and chemicals were from Sigma (Steinheim, Germany), and media from Biochrom (Berlin, Germany).

2.2 Total RNA isolation and cDNA preparation

Total RNA was harvested from cell lysates with the help of the Qiagen RNeasy kit. The 1 μg total RNA in 20 μl reaction mixture containing 500 μmol/L deoxyribonucleoside triphosphate (dNTP) mixture (GeneCraft, Münster, Germany), 250 ng random primer, and 200 U Superscript II RNase H^- (both from Invitrogen, Karlsruhe, Germany) was reversely transcribed to complementary DNA (cDNA), according to the Invitrogen's protocol.

2.3 Primer pair validation and qRT-PCR

To ensure maximum availability of the target gene, the 3′-end sequence of respective cDNAs was used for primer design. This was done with Primer Express software (Applied Biosystems, Darmstadt, Germany) and the resultant primer pairs were purchased from Proligo (Paris, France). Prior to their use in our in-depth qRT-PCR investigations, each primer pair was validated by titrating respective cDNA template at eight serial 1:5 dilutions in the PCR experiment. From the plot of cross threshold (C_t) values obtained against logarithmic serial concentrations of cDNA template, a straight line was constructed, defining the "linear dynamic range" of a cDNA template concentration for a specific primer pair. Within this concentration range of the template, respective primer pair affords correct C_t values in qRT-PCR (Applied Biosystems User Bulletin). From the slope m of the straight line defining the linear dynamic range of concentrations of a given cDNA template, the efficiency E (%) for respective primer pair was calculated according to $E=10^{-1/m}-1$ (Deprez *et al*., 2002). Primer pairs were redesigned for those efficiency values below 90% and above 110%. Higher efficiencies were caused by intra-primer hybridisation due to unspecific intercalation of SYBR Green with any double-stranded DNA. For all target genes investigated in this study, optimum efficiencies between 94% and 105% for respective primer pairs were obtained as shown in Table 1.

qRT-PCR was carried out in a 96-well GeneAmp 5700 Sequence Detection System using SYBR Green PCR Master Mix. Cycling parameters were according to Applied Biosystems' standard conditions. The reaction mixture (25 μl) contained diluted cDNA (equivalent to 25 ng total RNA), 200 nmol/L forward and reverse primer mixtures (Table 1), and 12.5 μl SYBR Green PCR Master Mix.

2.4 Data analysis

Of the three housekeeping genes tested, i.e., glyceraldehyde phosphate dehydrogenase (GAPDH), ubiquitin, and β-actin (Table 2), β-actin was found to be most stable under the experimental conditions employed. Consequently, comparative ΔC_t values for mRNA levels of target genes in HepG2 cells were calculated with reference to β-actin mRNA.

Table 1 Primer pairs used for optimized qRT-PCR and their efficiencies and linear ranges of cDNA templates

Gene	Primer pair	Dynamic linear range (ng cDNA)	Primer pair efficiency (%)	GenBank accession No.
β-Actin	F-5′-CGTCCACCGCAAATGCTT-3′ R-5′-GTTTTCTGCGCAAGTTAGGTTTTGT-3′	62.50–0.02	105	NM_001101
PPARα	F-5′-ATCCCAGGCTTCGCAAACTT-3′ R-5′-CATGGCGAATATGGCCTCAT-3′	62.50–0.10	101	NM_005036
PPARβ	F-5′-CGTACGATCCGCATGAAGCT-3′ R-5′-CTGGCACTTGTTGCGGTTCT-3′	62.50–0.10	92	NM_006238
PPARγ	F-5′-CCAAGGCTTCATGACAAGGG-3′ R-5′-GCAAACTCAAACTTGGGCTCC-3′	62.50–0.50	95	NM_138711
FAT/CD36	F-5′-GGAAAATGTAACCCAGGACGC-3′ R-5′-GATAGTGAAGGTTCGAAGATGGCA-3′	62.50–0.50	99	NM_000072
GLUT-2	F-5′-GAGTTGGCGCTGTAAACATGG-3′ R-5′-GCACAAGTCCCACTGACATGAA-3′	62.50–0.05	90	NM_000340
L-FABP	F-5′-TGAAGGCAATCGGTCTGCC-3′ R-5′-TCCCATTCTGCACGATTTCC-3′	12.50–0.02	94	NM_001443
ACOX-1	F-5′-CCAAGCTTTCCTGCTCAGTGTT-3′ R-5′-CCCCCAGTCCCTTTTCTTCA-3′	62.50–0.50	100	NM_007292
PBE	F-5′-GGGTAGGATTCACAAACC-3′ R-5′-GGTACGTGGTTCAATGTG-3′	31.25–0.25	99	NM_001966

3′-Ends of human target genes were used for primer design. Melting temperature (T_m) value was set as (60±1) °C and 200 nmol/L of forward (F) and reverse (R) primers were used per quantitative PCR reaction

Table 2 Impact of DMSO (solubilizer of agonists) on the induction of selected house keeping genes

Housekeeping gene	$2^{-\Delta\Delta C_t}$	
	Untreated	DMSO-treated
β-Actin	15.19±0.08	15.14±0.05
GAPDH	19.84±0.16	17.65±0.17
Ubiquitin	19.31±0.15	18.51±0.06

HepG2 cells were either treated with 1% DMSO or untreated. Data are expressed as mean±standard deviation (SD) of minimum three independent cell samples

Lower C_t values mean higher expression levels, and therefore we convert these values to $15-\Delta C_t$ values (here 15 is the C_t (nth PCR cycle at which plateau is obtained) value of β-actin as we obtained in our assay system, the standard housekeeping gene used for fold induction in this study), allowing display of higher values for higher expression levels. ΔC_t is the difference obtained by subtracting the C_t value of β-actin (15) from that of the candidate gene (it is usually higher than C_t values of housekeeping genes). Fold inductions in response to agonist administration to HepG2 cells refer to respective ΔC_t values of DMSO control (negative control, no FA treatment of cells), i.e., $\Delta C_{t,\,\text{agonist}}-\Delta C_{t,\,\text{DMSO}}$. Such values ($\Delta\Delta C_t$) can be converted to "relative expression" by the calculation of $2^{-\Delta\Delta C_t}$ values (Applied Biosystems Bulletin), where 1.0 means no regulation, i.e., values above 1.0 relate to induction, and values below 1.0 show repression (to demarcate this, a line is drawn in the Figs. 2 and 3 at Level 1).

3 Results and discussion

The choice of the human HepG2 cell culture is dictated by its fully preserved protein ensemble for carrying out PPAR-targeted FA signaling (Wolfrum *et al*., 2001). It is generally considered as a good model for the understanding of liver metabolism, as it is the natural habitat for the lipid transporters and enzymes whose expressions are regulated via PPAR activation (Nguyen *et al*., 2008). We have elaborated an optimized HepG2 cell-based transactivation system (Benjamin *et al*., 2005). We have shown that in vitro binding of 9-CLA and 10-CLA and their precursor FAs by PPAR subtypes did not correlate with their transactivation potentials, the most dramatic observation being that affinity of LA for PPARβ was almost zero, despite having a substantial transactivation potential for this subtype, in fact, close to that of 9-CLA (Benjamin *et al*., 2005). Aiming at the present study, the use of the HepG2 cell model was straight-forward in comparison to their transactivation potentials (Benjamin *et al*., 2005) and also in comparison to the agonists themselves.

One first prerequisite for optimized experimental conditions was the design of validated primers for qRT-PCR as described in materials and methods. The other was the choice of the right housekeeping gene for reference. Among three housekeeping genes tested, including the very commonly used GAPDH,

β-actin was proved to be the best housekeeper (Table 2). Our study revealed that "DMSO control" (no agonist dissolved in DMSO) had a significant impact on the expression of most of the candidate genes as compared to housekeeper β-actin gene, where L-FABP and PBE were strongly upregulated (Fig. 1). Some literature reports exist, describing DMSO as an inducer of the differentiation of cell lines, such as preosteoblast MC3T3-E1 (Cheung *et al*., 2006) and HL 60 cells (Yu *et al*., 2008), or as an inducer of expression of a gene encoding β-galactosidase fusion protein in stably transfected CHO cells (Liu *et al*., 2001). Masson *et al*. (2008) showed that DMSO induced interferon-γ (IFN-γ) and granzyme B expression in hepatic NKT and NK cells. Consequently, we subtracted the effect of DMSO as shown in Fig. 1 from the respective overall values for the expression of the candidate genes to arrive at the net effect, thus avoiding false positive results.

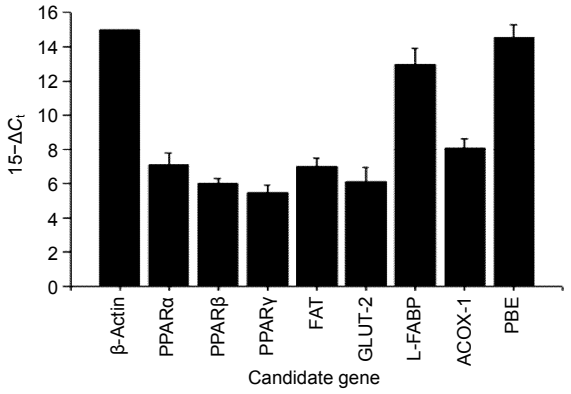

Fig. 1 Expression profile of genes in HepG2 cells
DMSO was used as control. Here 15 is the C_t (*n*th PCR cycle) value (plateau of PCR products) of β-actin, the standard housekeeping gene used for fold comparing induction in this study. C_t values of candidate genes were lower than that of β-actin, i.e., took more PCR cycles to reach the plateau. ΔC_t is the difference obtained by subtracting the C_t value of β-actin (15) from that of the candidate gene (it is usually higher than the C_t value of house keeping gene). Data are expressed as mean±SD of minimum three independent cell samples

3.1 Impact of agonists on expression of PPAR subtype mRNAs

Results showed that both 9-CLA and 10-CLA have induced all the PPAR subtypes equally but in a weak manner (~0–0.5-fold upregulation) with significantly higher induction of PPARγ (Fig. 2). Of the precursors of CLA tested, only LA significantly induced PPARβ and PPARγ (~1.0-fold upregulation). However, these induction values of PPAR subtypes are not in agreement with those values obtained in reporter gene assay (mechanistic condition) as reported by Benjamin *et al*. (2005) and Clement *et al*. (2002) for human PPARs in a HepG2 model. PPARα was transactivated by these CLAs to the highest level (~2.5-fold), PPARβ showed a medium activation, and PPARγ showed a weak action. This indicates that fold inductions of PPAR subtypes, due to the same agonists in reporter gene assay and as described in this study, were in reverse order and magnitude. In natural cellular environments (as in this study and in vivo), endogenous PPAR ligands like lipoprotein lipase (LPL) are known to have a selective effect on PPAR (PPARα>>PPARβ>PPARγ), apart from its enzymatic action (Ruby *et al*., 2010). This further hints that endogenous ligands like LPL have a higher preference for PPARα and exogenous ligands (as in the present study) show more preference for PPARγ, and thus PPARγ was more upregulated than PPARα and PPARβ, as found in this study.

Expression data from other studies also substantiate the induction profile comparatively (as above) lower than that found in transactivation (Clement *et al*., 2002; Benjamin *et al*., 2005). For instance, prolonged feeding of barrows with a CLA mixture insignificantly induced PPARα, but significantly upregulated PPARγ in the adult muscles (~0.5-fold relative expression) (Meadus, 2003), which is in corroboration to our data for PPARγ (Fig. 2c). McNeel *et al*. (2003) also observed slight upregulation of PPARγ in cultured human preadipocytes treated with CLAs. In a DNA microarray using Affymetrix GeneChip experiment, Fujiwara *et al*. (2003) have demonstrated that RNA samples of HepG2 cells fed with OA, arachidonic acid, eicosapentaenoic acid or docosahexaenoic acid resulted in a very weak induction (even repression) of PPARα.

Further, it has to be noted that the PPAR-specific drug BZF (positive control) did not show any significant effect on PPARs (Fig. 2). This agonist was found to modulate various functions like adipogenesis, glucose control, lipid metabolism, and vasculature, by increasing the expression of specific PPARs, depending on the physiological requirements (Benjamin and Spener, 2009). In fact, the effects of CLA and precursor FAs were generally more than that of the

control BZF. It seems that, even a marginal or 0.5-fold upregulation of PPARs would be enough to overexpress the target PPRE-bearing genes involved in lipid transport (e.g., FAT, GLUT-2, and L-FABP) and metabolism (e.g., ACOX-1 and PBE) at the cellular environment, which would enhance the physiological effects.

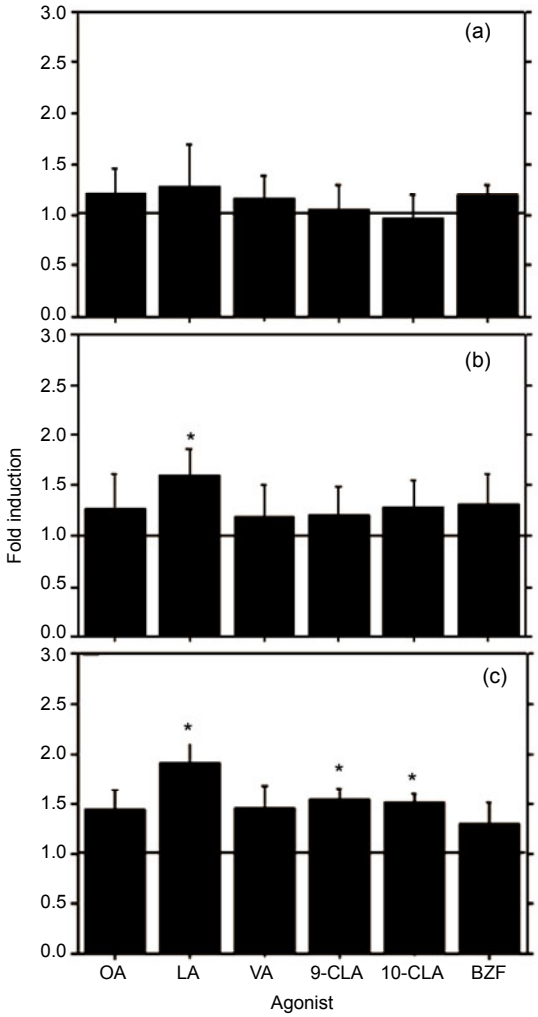

Fig. 2 Induction profiles of PPARs in HepG2 cells treated with lipid agonists: PPARα (a), PPARβ (b), and PPARγ (c)

The agonists (100 μmol/L in BSA) were OA, LA, VA, 9-CLA, 10-CLA, or the control BZF. Candidate genes tested were PPAR subtypes α, β, and γ. Fold inductions in response to agonist administration to HepG2 cells refer to respective ΔC_t values of DMSO control (negative control, no FA treatment of cells), i.e., $\Delta C_{t,\ agonist} - \Delta C_{t,\ DMSO}$. Compared to DMSO control, fold inductions at and above 1.5-fold were found to be significant ($P<0.05$). Symbol '*' denotes significant upregulation of the candidate gene against DMSO control by Student's *t*-test ($P<0.05$). Data are expressed as mean±SD ($n=3$)

3.2 Impact of agonists on expression of transporter mRNAs

All agonists tested in this study showed, on an average, 0.5–2.0-fold upregulation of all the PPRE-bearing transporter genes investigated herein, viz., FAT, GLUT-2, and L-FABP (Fig. 3). Fig. 3 further

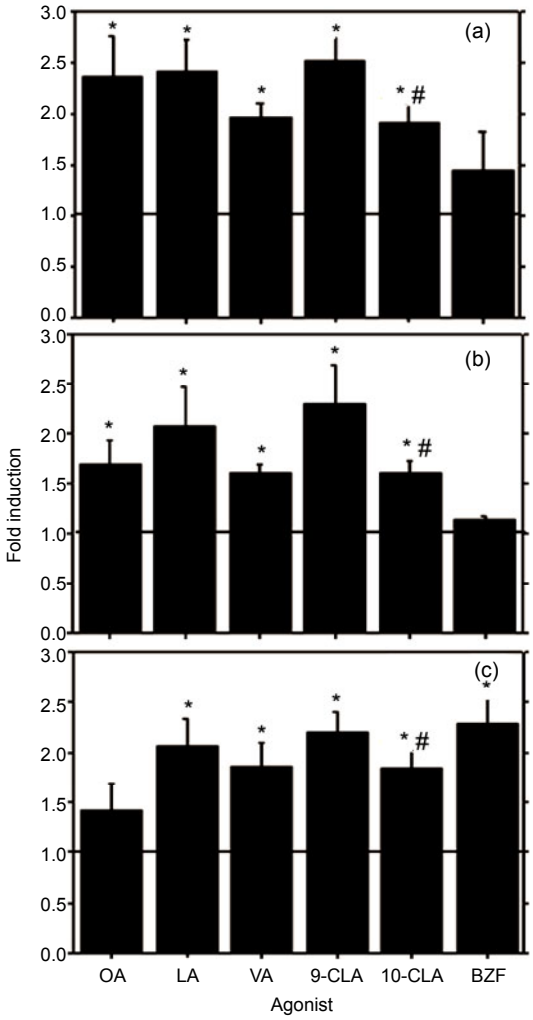

Fig. 3 Induction profiles of PPRE-bearing transporter genes in HepG2 cells treated with agonists: FAT (a), GLUT-2 (b), and L-FABP (c)

The agonists (100 μmol/L in BSA) were OA, LA, VA, 9-CLA, 10-CLA, or BZF. Candidate genes tested were FAT, GLUT-2, and L-FABP. Fold inductions in response to agonist administration to HepG2 cells refer to respective ΔC_t values of DMSO control (negative control, no FA treatment of cells), i.e., $\Delta C_{t,\ agonist} - \Delta C_{t,\ DMSO}$. Compared to DMSO control, fold inductions at and above 1.5-fold were found to be significant ($P<0.05$). Symbol '*' denotes significant upregulation of the candidate gene against DMSO control, and '#' against 10-CLA denotes significant difference from corresponding 9-CLA values by Student's *t*-test ($P<0.05$). Data are expressed as mean±SD ($n=3$)

demonstrates that, in comparison to 10-CLA, 9-CLA significantly upregulated all these transporters (~0.5-fold higher). Among the precursor FAs, LA emerged as the best inducer, with comparable induction values for 9-CLA (~1.0–2.0-fold induction) (Fig. 3). However, BZF significantly induced only L-FABP (Fig. 3c), which was at par with the upregulation of transporter genes by 9-CLA. Using a cDNA microarray cum RT-PCR technique, Frederiksen *et al*. (2004) showed in a Sprague Dawley rat model (for high cholesterol diet induced dyslipidemia) that the PPAR-target drugs could upregulate the PPAR-target genes, and the fold induction profiles by fenofibrate (PPARα specific drug), NNC16-3058 (PPARβ specific drug) and rosiglitazone (PPARγ specific drug) were 0.4, 0.3, 1.4 for GLUT-2 and 3.0, 4.5, 0.8 for FAT, respectively. Moya-Camarena *et al.* (1999) have observed that L-FABP expression was significantly high (~1.0-fold) in 9-CLA fed rat hepatoma FaO cell line (relative to DMSO control at 24 h), and Meadus (2003) found that about 0.5-fold higher induction of adipocyte FABP (A-FABP) was significant on porcine muscle in vivo to increase lean muscles. Weldon *et al.* (2004) demonstrated that LA and CLAs significantly enhanced FAT mRNA expression in macrophages and macrophage-derived foam cells. From this, it is clear that our data and the data reported by others (Frederiksen *et al*., 2004) for the lipid transporters cited herein are in agreement and that even a 0.5-fold increase of a gene will impart gross physiological effects in vivo.

3.3 Impact of agonists on expression of peroxisomal mRNAs

As shown in Fig. 4, 9-CLA and 10-CLA imparted contrasting effects on ACOX-1 and PBE, whose transcripts are involved in lipid metabolism, i.e., 9-CLA significantly downregulated the former, while the latter was upregulated (0.5-fold). The precursor FAs, viz., OA, LA, and VA, and the control BZF also upregulated ACOX-1 and PBE, and these values were comparable to those of transporter genes as reported in this study. In a clinical trial, Kuhnt *et al*. (2009) also showed that along with other 20 candidate genes, ACOX-1 and PBE are differentially expressed in human male and female subjects (gender-specific) upon VA consumption. As for transporter genes, Frederiksen *et al*. (2004) demonstrated that ACOX-1 and PBE were upregulated by the drugs fenofibrate, NNC16-3058, and rosiglitazone. Likewise, Moya-Camarena *et al*. (1999) reported a very significant upregulation (3.0-fold) of hepatic ACO in CLA-fed SD rats. Platt *et al*. (2007) demonstrated the differential actions of 9-CLA and 10-CLA on mineralised bone nodule formation; i.e., the 9-CLA increased the number (~11.0-fold) and size (~5.0-fold) of mineralized bone nodules at its 100 μmol/L concentration, while 10-CLA had no effect. This indicates that the CLAs play a major role in signal modulation rather than a nutritional role.

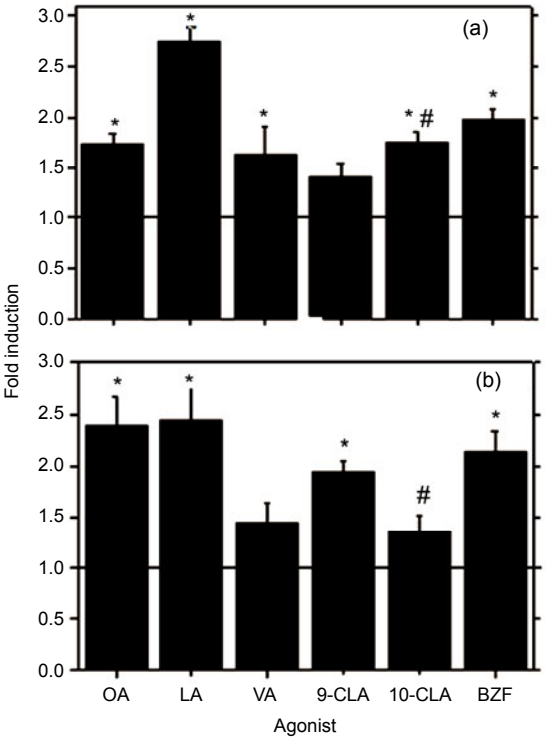

Fig. 4 Induction profiles of PPRE-bearing peroxisomal genes in HepG2 cells treated with agonists: ACOX-1 (a) and PBE (b)

The agonists (100 μmol/L in BSA) were OA, LA, VA, 9-CLA, 10-CLA, or BZF. Candidate genes tested were: acyl CoA oxidase-1 (ACOX1) and peroxisomal bifunctional enzyme (PBE). Fold inductions in response to agonist administration to HepG2 cells refer to respective ΔC_t values of DMSO control (negative control, no FA treatment of cells), i.e., $\Delta C_{t,\ agonist} - \Delta C_{t,\ DMSO}$. Compared to DMSO control, fold inductions at and above 1.5-fold were found to be significant ($P<0.05$). Symbol '*' denotes significant upregulation of the candidate gene against DMSO control, and '#' against 10-CLA denotes significant difference from corresponding 9-CLA values by Student's *t*-test ($P<0.05$). Data are expressed as mean±SD ($n=3$)

4 Conclusions

In conclusion, it is evident that drugs can specifically induce PPARs and certain PPAR-target genes at an extra-ordinarily higher level on animal models, yet a normal range of induction of these genes by CLAs and their precursors lies at a range of ~0.5–2.0-fold as shown by our group and others in related studies. At the cellular level in vivo, this fold induction would be more than enough to elicit all connected biochemical pathways to the maximum extent, beyond which untoward side effects may occur. Nevertheless, while calculating the actual fold induction of the candidate genes, the impacts of the solubilizer DMSO and the reference housekeeping gene should also be taken into account, which may cause false positive results. Briefly, the present study shows that CLAs and their precursor FAs upregulated the PPAR-target genes moderately, but at a significantly higher level than the PPAR subtypes, so as to elicit the required physiological effects. 9-CLA and 10-CLA show distinct influences on PPRE-bearing genes. All these effects indirectly show that CLAs are involved in lipid signalling by modulating the PPAR α, β, γ subtypes for the indirect activation of the PPAR-target genes, which may in turn be responsible for the supposed health effects of CLA.

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