Original Article

Differential Effects of Long-Chain Fatty Acids and Clofibrate on Gene Expression Profiles in Cardiomyocytes

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Background: The link between dietary fat and coronary heart disease has attracted much attention since the effect of long-chain fatty acids on gene transcription has been established. The aim of this study was to investigate the effects of long-chain fatty acids and clofibrate on mRNA levels of specific lipid metabolism-related genes and to determine their effects on global transcriptome levels in a cardiovascular cell-line.

Methods: After culturing P19CL6 cells with long-chain fatty acids or clofibrate, the expression levels of heart-type fatty-acid binding protein and peroxisome proliferators-activated receptors (PPARα,β,γ) were determined by reverse transcriptase-polymerase chain reaction. Additionally, global transcriptome profiles were compared using microarray analysis.

Results: Long-chain fatty acids significantly increased the abundance of PPARα and PPARγ. Moreover, microarray analysis showed that the effects of linoleic and α-linolenic acids, and clofibrate were similar but differed from those of palmitic and oleic acids.

Conclusion: These findings show that cellular responses to polyunsaturated fatty acids differ from those observed with saturated and monounsaturated fatty acids.

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Keywords: Cardiomyocyte • fatty acids • gene • microarray

Introduction

Coronary heart disease (CHD) is the leading cause of death in developed countries[1, 2] and its prevention and treatment continues to be a significant challenge.[3] The link between dietary fat and heart disease has been clearly established.[4,5] Saturated fatty acids (SFAs) are considered as atherogenic fat, whereas polyunsaturated fatty acids (PUFAs) are cardioprotective.[5–8] Many of the potentially beneficial effects of dietary PUFAs in preventing or inhibiting the progression of CHD are mediated through the control of gene transcription.[5,9] The regulatory function of long-chain fatty acids (LCFAs) on gene expression is achieved by controlling the abundance and activity of transcription factors such as sterol regulatory element-binding protein, liver X-receptor, and peroxisome proliferators-activated receptors (PPARα,β,γ).[10–13]

Several cell culture and animal model systems have been used to study the effects of LCFAs and fibrates at the gene expression level.[8,14–18] However, there are a limited suitable cardiovascular model systems available for the *in vitro* investigation of FA effects on cardiomyocytes.[19,20] The P19Cl6 cell-line used in this study is a subclone of P19 mouse embryonal carcinoma cells that has been reported to efficiently differentiate into cardiomyocytes upon exposure to dimethyl sulfoxide (DMSO).[21] Therefore, based on the effects of LCFAs and fibrates on the other cardiomyocyte model systems, it was hypothesized that the mRNA levels of lipid metabolism-related genes such as heart-type fatty acid-binding protein (H-FABP), PPARα, PPARβ, and PPARγ would be affected by these ligands in P19CL6 cells. In addition, since the

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effects of FAs differ based on their chain length and degree of unsaturation,[22,23] it was hypothesized that LCFAs would differentially affect the expression levels of these proteins. Furthermore, the effects of LCFAs and fibrates on global transcriptome levels were investigated in order to obtain a broader picture of the effects of these compounds on gene expression levels in the P19CL6 cell-line. Therefore, the aim of this study was to investigate the effects of LCFAs including palmitic (saturated FA), oleic (monounsaturated FA), linoleic (n-6 polyunsaturated FA), and α-linolenic (n-3 polyunsaturated FA) acids, as well as clofibrate on gene expression levels in P19CL6 cells.

Materials and Methods

Sigma-Aldrich Company Ltd. (Poole, UK) supplied 100× penicillin-streptomycin and DMSO. Fetal bovine serum (FBS), α-minimum essential medium (α-MEM), Trizol reagent, and phosphate-buffered saline (PBS) tablets were obtained from Invitrogen Corporation (Paisley, UK). Nested polymerase chain reaction (PCR) kit and agarose for gel electrophoresis were purchased from Promega Corporation (Southampton, UK) and MWG Biotech Ltd. (Milton Keynes, UK) supplied oligonucleotide primers. Ready-To-Go reverse transcriptase-PCR (RT-PCR) kit, Microarray Slides, Cy3-dCTP (labeled 2'-deoxycytidine 5'-triphosphates), and Cy5-dCTP fluorescent dyes were obtained from Amersham Biosciences (Chalfont St. Giles, UK). Corning Life Sciences (Corning B.V. Life Sciences, Schiphol-Rijk, Netherlands) supplied ProntoPlus microarray kit. RN*easy*® MinElute™ Cleanup kit, RN*ase*-free DN*ase*, and MinElute PCR purification kit were obtained from Qiagen Ltd. (Crawley, UK). Tissue culture plates were provided by Nunclon Products (Nunclon, Roskilde, Denmark) and 150 cm^2 peel-off flasks were obtained from Helena BioSciences Europe Ltd. (Sunderland, UK).

Methods

Cell culture

P19CL6 cells were purchased from Riken Cell Bank (Ibaraki, Japan),[21] and cultured in normal culture media containing α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin. To differentiate P19CL6 cells into cardiomyocytes, P19CL6 cells were cultured in 150 cm^2 peel-off flasks at a density of 2×10^6 cells per flask using 30 mL of a DMSO-containing medium (α-MEM/10% FBS/1% DMSO/1% penicillin-streptomycin) for 14 days. At the end of the day 14, medium was removed and cells were washed twice with 15 mL PBS and incubated in 25 mL of a serum-free medium (98% α-MEM/1% penicillin-streptomycin/ 1% DMSO) for 24 hours (day 15). The cells were then treated with media either containing bovine serum albumin (BSA), as carrier (98% α-MEM/ 1% penicillin-streptomycin/ 1% DMSO/ 0.15 mM BSA), BSA-FA complex (98% α-MEM/ 1% penicillin-streptomycin 1% DMSO/0.15 mM BSA with 0.4 mM FA), or BSA-clofibrate complex (98% α-MEM/1% penicillin-streptomycin/ 1% DMSO/0.15 mM BSA with 0.4 mM clofibrate) for 24 hours.[21,24] Finally, the medium was aspirated off and cells were washed with 15 mL PBS. Cell lysate was obtained by adding 15 mL of Trizol reagent (1 mL per 10 cm^2 culture area) and stored at –80°C until RNA extraction.

Preparation of BSA/FA complex

An appropriate amount of BSA was dissolved in a serum-free medium to obtain a concentration of 0.15 mM BSA. In the next step, FA or clofibrate powder was dissolved in 100 mM NaOH to prepare a 100 mM stock solution[24]; the latter was then diluted at the ratio of 1:250 with serum-free medium containing 0.15 mM BSA to achieve a final concentration of 0.4 mM (or clofibrate).

Total-RNA isolation and purification

Total RNA was isolated by the direct addition of Trizol reagent to the cells (1 mL per 10 cm^2 of cell culture area) and proceeding manufacturer's instructions. For microarray experiments the total-RNA samples were treated with DN*ase*-I enzyme to remove potential genomic DNA contamination and purified using RN*easy* MinElute Spin columns. Finally, a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, USA) was used to determine total-RNA concentration. The ratio of absorbance at 260 and 280 nm (A260/A280) was used to assess purity of RNA samples with a ratio of 1.8 – 2.0 generally being accepted as pure RNA.[25,26]

Determination of mRNA levels

To determine gene expression levels, the levels

of specific mRNAs were determined by RT-PCR using relevant gene-specific forward and reverse primers. In some experiments, RT-PCR products were reamplified by nested PCR to achieve higher cDNA levels.[27,28] Primers for both RT-PCR and nested PCR techniques were designed using Primer-3 software (Primer3™, Whitehead Institute for Biomedical Research, Cambridge, UK). In order to get semiquantitative values of gene expression levels, agarose gels (2%) containing ethidium bromide were used. PCR products were then separated by electrophoresis at 100 V and the gels photographed using an ImageMaster VDS UV-light camera (Amersham Pharmacia Biotech, San Francisco, USA). The photographic images of gels were then digitized using the Scion Image program (ScionImage™, Scion Corporation, Maryland, USA) and gene expression levels were determined relative to the expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[29]

Microarray experiments

Microarray experiment was designed as dual-hybridization optimal interwoven loop (Figure 1) using an online microarray experimental design program, MARIE software (MARIE™, Biotechnology and Biological Sciences Research Council, Swindon, UK).[30–32]

Microarray experiment was carried out in a step-by-step procedure including cDNA synthesis and labelling by fluorescent dye,[25] purification of labeled-cDNA, and hybridization of labeled-cDNA on arrays, as indicated in ProntoPlus kit instructions. Images were acquired by scanning of arrays using an Affymetrix 428 scanner (Affymetrix, Santa Clara, USA). BlueFuse software (BlueGenome Ltd., Great Shelford, UK) was used for image analysis and the microarray data further analysed using Gene Spring-7 software (Silicon Genetics, Redwood City, USA). After normalization and filtering of microarray data, linear modeling (LLAMA; Live Linear Analysis of MicroArray) was performed (LLAMA™, Biotechnology and Biological Sciences Research Council, Swindon, UK) in order to convert the data from the loop experiment into a linear model.[33-35] The data produced by linear modeling were imported into a web-based clustering program (Cluster™, Lawrence Brekely National Laboratory, Brekely, USA)[36] to generate a pair-wise linkage cluster. Analysis formed a hierarch-

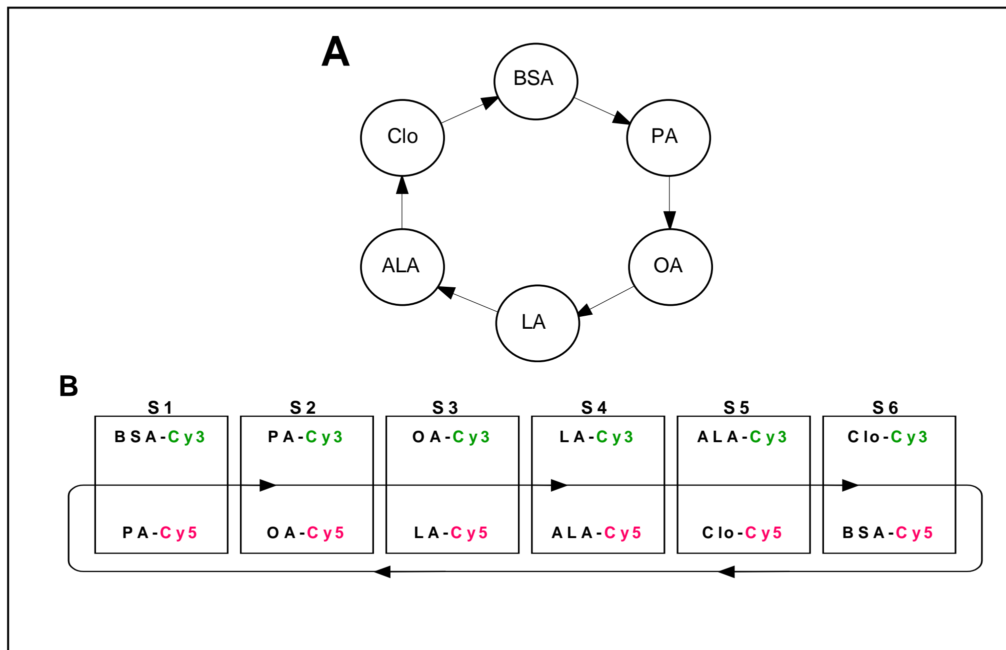

Figure 1. Experimental loop design to compare global transcriptome levels in P19CL6 cells cultured with LCFAs or clofibrate. (A) Loop design showing the six samples (nodes) to be compared. The sample at the start of the arrow was labeled with Cy3 and the "target" sample with Cy5. (B) Samples and Cy-dye labeling used on different microarray slides, based on the loop design. S1-S6=slide numbers. ALA=α-linolenic acid; BSA=bovine serum albumin; Clo= clofibrate; LA=linoleic acid; OA=oleic acid; PA=palmitic acid.

ical cluster based on the phylogenetic algorithm, which was then visualized with TreeView software (TreeView™, Lawrence Brekely National Laboratory, Brekely, USA).[36]

Results

In order to determine the effects of LCFAs and fibrates on gene expression levels, P19CL6 cells were cultured with 0.4 mM LCFAs or clofibrate, as discussed in the Materials and Methods. The mRNA expression of H-FABP, PPARα, PPARβ, and PPARγ in P19CL6 cells was detected by RT-PCR followed by nested PCR techniques. Semiquantitative gene expression levels, relative to GAPDH expression, were then calculated (Table 1). A One-way ANOVA analysis was conducted to explore the impact of LCFAs or clofibrate on gene expression. The expression of H-FABP and PPARβ did not display significant changes with LCFAs or clofibrate (Table 1). However, LCFAs had a significant effect ($P<0.05$) on the expression of PPARα and PPARγ in P19CL6 cells (Table 1). In addition, levels of PPARα and PPARγ mRNA were significantly increased with all the LCFAs, but not with clofibrate (Table 1). Although this semi-quantitative data may be indicative of the underlying biologic response, it should be viewed as preliminary, and therefore, in order to get a wider prospective on the effects of LCFAs and clofibrate in P19CL6 cells, microarray analysis was used to investigate global transcriptome profiles in the cells cultured with these ligands.

The data from microarray experiment were converted into a linear model, which was subsequently imported into Gene Cluster program. Complete-linkage hierarchical cluster analysis of microarray data showed that expression levels in cells treated with linoleic acid (LA) or α-linolenic acid (ALA) were similar and closely related to the general effect observed with clofibrate-treated cells (Figure 2). A similar pattern was also shown with principal component analysis (PCA) confirming that LA, ALA, and clofibrate were closely related to each other (Figure 3) but differed from that of palmitic acid (PA) and oleic acid (OA) (Figures 2 and 3).

Discussion

The study of mammalian gene expression is often carried out at the level of mRNA and relies upon techniques for identification and quantitation of mRNA species coding for specific proteins. Several methods have been developed for this purpose, each offering distinct advantages and disadvantages.[37] The most common techniques to measure gene expression involve Northern hybridization analysis, ribonuclease protection, and RT-PCR.[37,38] The latter has become the benchmark for detection and quantification of RNA targets and is utilized increasingly in clinical diagnostic assays.[38,39] However, despite the advantages and the greater accuracy of real-time RT-PCR in quantification of a distinct gene or a disease-specific diagnostic marker,[38] semi-quantitative RT-PCR methods are still widely used for many purposes.[40]

Here, in order to determine the effect of LCFAs and clofibrate on gene expression levels in P19CL6 cells, the mRNA expression levels of lipid metabolism-related genes, i.e., H-FABP, PPARα, PPARβ, and PPARγ were initially investigated by semiquantitative PCR analysis in a focused study, while global transcriptome levels were subsequently studied by microarray analysis.

A semiquantitative estimation of gene expression levels, using RT-PCR, in P19CL6 cells cultured with LCFAs or clofibrate (0.4 mM) showed no significant difference in the relative expression levels of H-FABP (Table 1). Previous

Table 1. Comparison of relative H-FABP, PPARα, PPARβ, and PPARγ gene expression levels in P19CL6 cells cultured with different LCFAs or clofibrate.

Gene	Gene expression relative to GAPDH (mean±SD)					
	BSA	PA	OA	LA	ALA	Clo
H-FABP	1.22±0.11	1.15±0.18	1.27±0.12	1.22±0.27	1.27±0.23	1.23±0.16
PPARα	1.27±0.14	1.70±0.40[a]	1.89±0.19[b]	1.99±0.16[b]	2.04±0.24[b]	1.49±0.15
PPARβ	0.97±0.37	1.09±0.16	1.16±0.23	1.37±0.33	1.35±0.17	1.11±0.17
PPARγ	1.21±0.23	1.78±0.21[a]	1.96±0.22[b]	2.14±0.26[b]	1.80±0.26[a]	1.24±0.20

Data are shown as mean±standard deviation and significant differences in gene expression levels relative to BSA treatment were indicated as [a]$P<0.05$, and [b]$P<0.001$. ALA=α-linolenic acid; BSA=bovine serum albumin; Clo=clofibrate; LA=linoleic acid; OA=oleic acid; PA=palmitic acid.

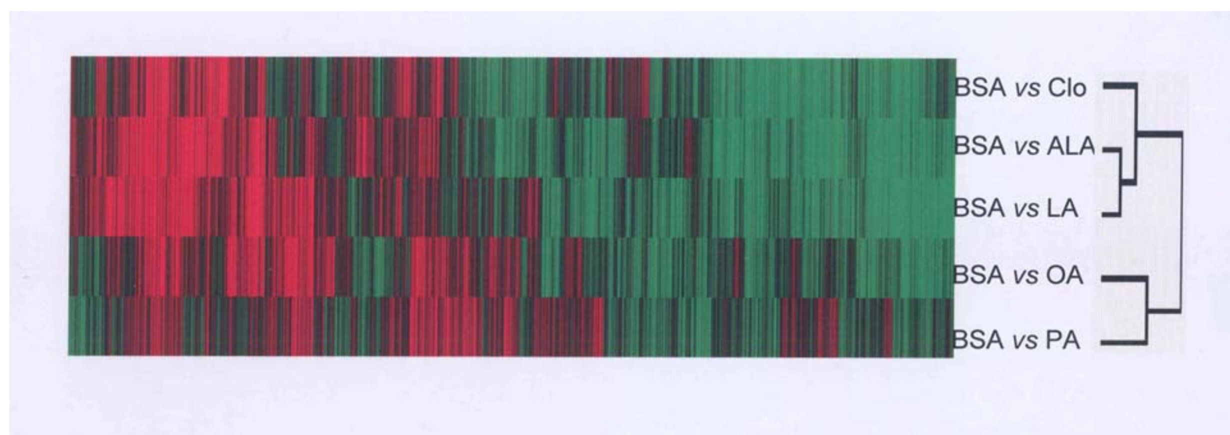

Figure 2. Hierarchical cluster analysis of mRNA expression levels in microarrays comparing the P19CL6 cells cultured with LCFAs and clofibrate. The comparisons were clustered according to the degree of pair-wise similarity. The color gradient represents green for down-regulation and red for up-regulation. Each gene is represented by a single row and comparisons are demonstrated by columns. ALA=α-linolenic acid; BSA=bovine serum albumin; Clo=clofibrate; LA= linoleic acid; OA=oleic acid; PA=palmitic acid.

studies have shown that LCFAs or fibrates up-regulate H-FABP in cardiomyocytes.[41-43] However, other studies have shown no changes in H-FABP mRNA after a high-fat diet.[42,44] Similar contradictory observations have also been reported on the expression of H-FABP in skeletal muscle cells, indicating no significant response in cellular H-FABP content to high-fat diet for one week,[44,45] whereas long-term (four weeks) supplementation significantly increased expression of H-FABP mRNA.[45] Therefore, the nonresponse of H-FABP expression levels, as observed in our study, can probably be explained by the relatively short exposure time (24 hours). This leads to the conclusion that H-FABP gene expression responds slowly to LCFAs and clofibrate, and incubations for longer than 24 hours would probably be required before any effects of these ligands on H-FABP mRNA expression would be observed.

P19CL6 cells cultured with LCFAs or clofibrate also showed no significant changes in the relative expression level of PPARβ, whereas the expression of PPARα and PPARγ was significantly increased in the presence of LCFAs, regardless of the chain length and degree of unsaturation (Table 1). Therefore, the observations made in this study are in agreement with previous studies, which showed that dietary fat and fibrates increased PPARα and PPARγ expression levels in different tissues.[46-49] Unlike the significant increase observed in PPARα and PPARγ expression levels, incubation of P19CL6 cells with LCFAs or clofibrate did not affect the expression of PPARβ (Table 1). This observation is consistent with previous reports that showed the ingestion of dietary fat had no effect on PPARβ expression levels, as shown in rat colonic mucosa and human endothelial cells.[50,51]

In this study, P19CL6 cells cultured with clofibrate showed no statistically significant change in the expression levels of the PPAR isoforms (Table 1), although, the induction of PPARα mRNA by clofibrate has been shown in other model systems.[52–54] The nonresponse of PPARα gene expression level observed here can probably be explained by the short exposure time (24 hours),[55] because long-term incubations have been mainly used in other studies.[52,54]

In addition to the effect of LCFAs and clofibrate on H-FABP, PPARα, PPARβ, and PPARγ expression level, the effects of these ligands on global transcriptome levels in the P19CL6 cell-line were also investigated by cDNA microarray analysis. LA, ALA, and clofibrate showed similar effects, as shown by cluster analysis (Figure 2) and PCA (Figure 3), whereas PA and OA had a different effect (Figures 2 and 3). These observations are in agreement with previous studies that showed dietary SFAs and PUFAs had different effects on gene expression.[56-59] This could be explained by differences in the affinity of SFA and PUFA to activate nuclear receptors.[11,60,61] The effect of clofibrate on global transcriptome profiles was also more closely related to LA and ALA than PA or OA (Figures 2 and 3). Therefore, the effect of PUFAs were similar to that of clofibrate, but differed from that of SFA which is a monounsaturated fatty acid.

In conclusion, both RT-PCR and microarray experiments showed that culturing P19CL6 cells

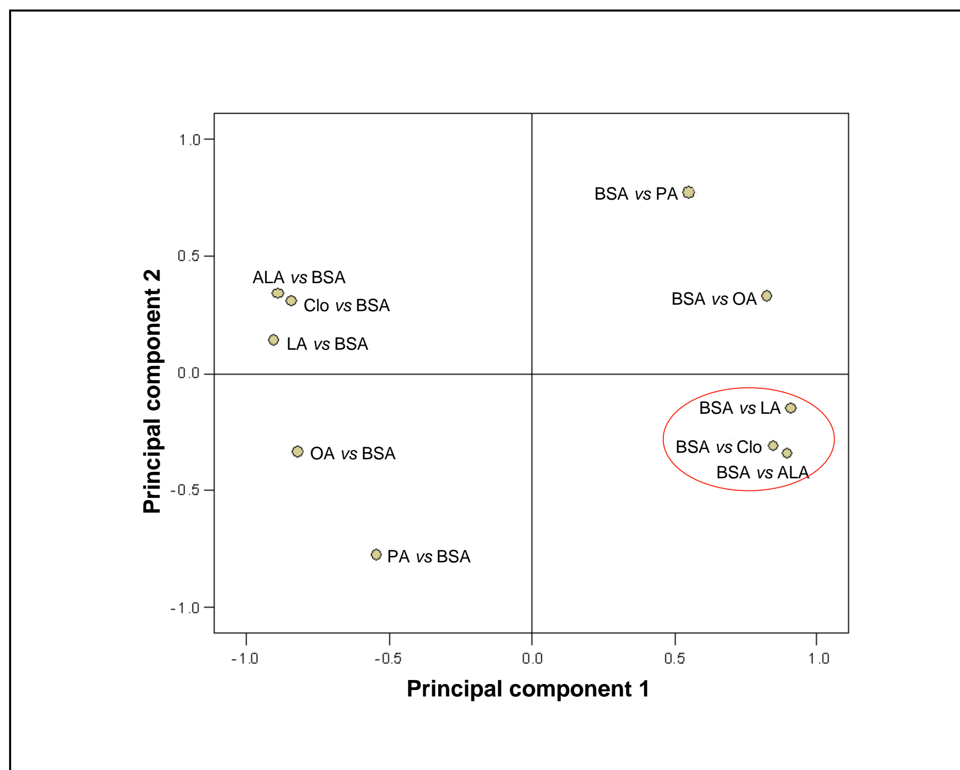

Figure 3. Principal component analysis (PCA) of global transcriptome levels in P19CL6 cells cultured with LCFAs or clofibrate. The red circle shows close relation between the samples. PC1 and PC2 represent 66% and 19% of total variance, respectively. ALA=α-linolenic acid; BSA=bovine serum albumin; Clo=clofibrate; LA=linoleic acid; OA=oleic acid; PA=palmitic acid.

with LCFAs or clofibrate affected gene expression. PCR experiments confirmed that LCFAs affect the abundance of PPARα and PPARγ, but not PPARβ or H-FABP under the conditions used. Microarray experiments, on the other hand, showed that the effects of LA, ALA, and clofibrate were similar but differed from those of PA and OA. In addition, the effect of clofibrate was similar to those of PUFAs.

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