

Bioinformatic profiling of the transcriptional response of adult rat cardiomyocytes to distinct fatty acids[§]

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Abstract Diabetes mellitus, obesity, and dyslipidemia increase risk for cardiovascular disease, and expose the heart to high plasma fatty acid (FA) levels. Recent studies suggest that distinct FA species are cardiotoxic (e.g., palmitate), while others are cardioprotective (e.g., oleate), although the molecular mechanisms mediating these observations are unclear. The purpose of the present study was to investigate the differential effects of distinct FA species (varying carbon length and degree of saturation) on adult rat cardiomyocyte (ARC) gene expression. ARCs were initially challenged with 0.4 mM octanoate (8:0), palmitate (16:0), stearate (18:0), oleate (18:1), or linoleate (18:2) for 24 h. Microarray analysis revealed differential regulation of gene expression by the distinct FAs; the order regarding the number of genes whose expression was influenced by a specific FA was octanoate (1,188) > stearate (740) > palmitate (590) > oleate (83) > linoleate (65). In general, cardioprotective FAs (e.g., oleate) increased expression of genes promoting FA oxidation to a greater extent than cardiotoxic FAs (e.g., palmitate), whereas the latter induced markers of endoplasmic reticulum and oxidative stress. Subsequent RT-PCR analysis revealed distinct time- and concentration-dependent effects of these FA species, in a gene-specific manner. For example, stearate- and palmitate-mediated *ucp3* induction tended to be transient (i.e., initial high induction, followed by subsequent repression), whereas oleate-mediated induction was sustained. **¶** These findings may provide insight into why diets high in unsaturated FAs (e.g., oleate) are cardioprotective, whereas diets rich in saturated FAs (e.g., palmitate) are not.—Lockridge, J. B., M. L. Sailors, D. J. Durgan, O. Egbejimi, W. J. Jeong, M. S. Bray, W. C. Stanley, and M. E. Young. **Bioinformatic profiling of the transcriptional response of adult rat cardiomyocytes to distinct fatty acids.** *J. Lipid Res.* 2008. 49: 1395–1408.

Supplementary key words β -oxidation • endoplasmic reticulum stress • gene expression

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Fatty acids (FAs) are more than just a substrate for oxidative metabolism. Fatty acids (and their derivatives) have been shown to influence transcription, translation, enzymatic activities, cellular signaling, ion homeostasis, membrane fluidity, and cell survival (1–7). It is therefore not surprising that cells have evolved mechanisms facilitating a balance between FA availability and rates of FA utilization. For example, when exposed to elevated circulating FAs, organs such as the heart, skeletal muscle, and liver respond by inducing genes that promote FA utilization, a response involving a number of distinct molecular mechanisms (8). One such mechanism involves a family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs) (9). Three PPAR isoforms are known, of which PPAR α and PPAR β/δ are highly expressed in tissues exhibiting relatively high rates of FA oxidative metabolism (e.g., liver, skeletal muscle, and heart), whereas PPAR γ is highly expressed in lipogenic tissues (e.g., adipose tissue and liver) (10). Upon activation through direct binding of FAs, PPARs, in combination with their heterodimerization partners, retinoid X receptors (RXRs), induce genes promoting FA utilization (9, 11). For example, prolonged increases in circulating FA levels (e.g., fasting, high-fat feeding, diabetes mellitus) are associated with increased expression of β -oxidation enzymes [and increased rates of FA oxidation (FAO)] in peripheral tissues such as liver, skeletal muscle, and heart, which is attenuated in PPAR α - and PPAR β/δ -null mice (12–15). It should be noted that PPARs are not the only mechanism by which FAs influence gene expression. Several additional mechanisms exist, which include alterations in LXR, CHREBP, HNF-4 α , and TLR4 activity (8). However, to date, studies on these non-PPAR-mediated mechanisms have focused primarily on extra-cardiac tissues (e.g., liver).

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Important previously published studies investigating the direct effects of FAs on myocardial gene expression have utilized neonatal cardiomyocytes, wherein the effects of a single FA species on a limited number of candidate genes are typically investigated (16, 17). Limitations to this approach include the observation that neonatal cardiomyocytes differentially express many of the known nuclear receptors (e.g., PPAR α) and coactivators (e.g., PGC1) involved in responsiveness of the myocardium to FAs, compared with adult hearts (18). In addition, not all FA species function in a biologically identical manner. Variations in carbon chain length and degree of saturation dictate not only the metabolic fate of the FA but also its biological influence on the cell. For example, long-chain saturated FAs exhibit increased lipotoxic properties (e.g., palmitate-induced apoptosis), an effect that can be attenuated by the long-chain monounsaturated FA oleate (19, 20). To date, determination of the direct effects of distinct FA species on adult cardiomyocytes has not been approached in a systematic, unbiased manner. The purpose of the present study was therefore to investigate the global transcriptional response of adult rat cardiomyocytes (ARCs) to five distinct FA species, which varied in carbon chain length and degree of saturation. We hypothesized that novel insight into the mechanisms of cardioprotective versus cardiotoxic effects might be revealed. Here we report that long-chain unsaturated FAs (e.g., oleate and linoleate) induce genes known to promote β -oxidation, whereas long-chain saturated FAs (e.g., palmitate and stearate) induce markers of endoplasmic reticulum (ER) and oxidative stress.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River, Fredrick, NY; 200 g initial weight) were housed at the Animal Care Center of the University of Texas Health Science Center at Houston under controlled conditions (23 \pm 1°C; 12 h light/12 h dark cycle), and received standard laboratory chow and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston.

Isolated ARCs

Isolated ARCs were prepared using protocols previously described (21, 22). Freshly isolated cardiomyocytes were cultured overnight in serum-free DMEM-containing laminin-coated plates. The cells were next (i.e., at time 0) challenged with one of five distinct FAs [octanoate (8:0), palmitate (16:0), stearate (18:0), oleate (18:1), and linoleate (18:2)] at a final concentration of 0.1 mM, 0.2 mM, or 0.4 mM. All FAs were conjugated to defatted BSA, which was present in the culture medium at a final concentration of 1%. Control cells were cultured in the presence of 1% BSA alone. BSA and BSA-FA conjugates were dialyzed overnight, sterile filtered, and the FA concentration confirmed by spectrophotometric assay (Wako). After 0, 3, 6, 12, 24, or 48 h of challenge, cardiomyocytes were harvested in TriReagent, and stored at -80°C prior to RNA isolation.

Microarray analysis

Microarray gene expression studies were performed using Sentrix BeadChips and the BeadStation system from Illumina, Inc. (San Diego, CA). Microarrays were performed according to the manufacturer's guidelines. Following extraction through standard procedures (23), total RNA was converted to cDNA by reverse transcription using ArrayScript reverse transcriptase and T7-(dT)24 primers, followed by second-strand synthesis to generate double-stranded cDNA (Ambion, Inc.; Austin, TX). After purification, the cDNA was converted to biotin-labeled cRNA, hybridized to a rat Ref-12 BeadChip array (Illumina, Inc.) and stained with streptavidin-Cy3 for visualization. The rat Ref-12 BeadChips contain sequences representing approximately 22,000 curated genes and putative expressed sequence tags, with an average of 30 replicates for each gene/transcript on each BeadChip. Quality standards for hybridization, labeling, staining, background signal, and basal level of housekeeping gene expression were verified for all BeadChips.

After the probe arrays were scanned, the resulting images were first analyzed using the BeadStudio software (Illumina, Inc.), which calculates the mean fluorescence signal across all replicates of each gene/transcript, along with the probability that the mean signal for each gene/transcript on the chip is greater than background (i.e., detection score). Genes/transcripts were defined as being significantly expressed above background when the average detection score across all replicates was greater than 0.75.

Quantitative RT-PCR

Quantitative RT-PCR of samples was performed using previously described methods (24, 25). Specific quantitative assays were designed from rat sequences available in GenBank. Taqman assays utilized for cytosolic thioesterase 1 (*cte1*), *cyclophilin*, malonyl-CoA decarboxylase (*mcd*), mitochondrial thioesterase 1 (*mte1*), pyruvate dehydrogenase kinase 4 (*pdk4*), and uncoupling protein 3 (*ucp3*) have been published previously (13, 22, 26, 27), and those for adipose differentiation-related protein (*adrb*) and pleckstrin homology-like domain a1 (*phlda1*) are reported in supplementary Table I. Standard RNA was made for all assays by the T7 polymerase method (Ambion) using total RNA isolated from rat hearts. The correlation between the C_t (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a 5-log range of RNA for all assays (data not shown). Quantitative RT-PCR data were normalized to *cyclophilin* expression, and expressed relative to the control group.

Statistical analysis

For the microarray study, differential expression of genes/transcripts, relative to control values, was calculated by one-way ANOVA, with correction for type I error using false-discovery rate (FDR) adjustment (SAS Corp.). Scheffe post hoc analysis was used to perform pairwise comparisons between treatment groups, for genes exhibiting significant main effects after FDR adjustment. Ontology analyses were performed in order to group genes from the final lists using the Onto-Tools package of ontology from Wayne State University (<http://vortex.cs.wayne.edu/projects.htm>). Normalized data have been submitted to the GEO archive, and are available at <http://www.ncbi.nlm.nih.gov/geo/>. For RT-PCR data, ANOVA was conducted to investigate the main effects of concentration separately for each FA and time, followed by post hoc pairwise comparisons. The significance level for post hoc pairwise comparisons was adjusted using a conservative Bonferroni approach. Stata version 8.0 (Stata Corp.; San Antonio, TX) was used to perform this analysis. The null hypothesis of no treatment effects was rejected at $P < 0.05$.

RESULTS

Genome-wide effects of distinct FAs on ARC gene expression

In an attempt to improve our understanding of the transcriptional response of ARCs to distinct FAs, gene expression profiling was performed through the use of microarrays. ARCs were therefore challenged with 0.4 mM octanoate, palmitate, stearate, oleate, or linoleate for 24 h, after which RNA was isolated and utilized for gene expression analysis; exposure of ARCs to 0.4 mM oleate for 24 h has previously been shown to elicit maximal effects on metabolic gene expression (13, 22). Control cells were treated in a manner identical to that used for the experimental groups, with the exception that no FA was added to the medium. Of the approximate 22,000 genes/transcripts interrogated through microarray analysis, approximately 11,500 were expressed in control ARCs. Supplementary Table II reports the number of genes differentially expressed in ARCs following challenge with distinct FA species. Somewhat surprisingly, of the five FA species investigated, challenging ARCs with the medium-chain FA octanoate resulted in the largest number (1,188) of differences in gene expression, relative to control cells. The saturated long-chain FAs palmitate and stearate influenced the expression of a similar number of genes compared with one another (590 and 740, respectively, relative to control), whereas the unsaturated long-chain FAs oleate and linoleate influenced expression of the smallest number of genes (83 and 65, respectively, relative to control). This sub-stratification based on the num-

ber of differentially expressed genes for medium-chain, saturated long-chain, and unsaturated long-chain FAs was also mirrored at the levels of induced versus repressed genes (see supplementary Table III). For all FAs investigated, approximately two-thirds of the differentially expressed genes were induced, and approximately one-third were repressed, compared with control cells. In addition, a high level of similarity was observed between the genes influenced by palmitate and stearate, and between oleate and linoleate (see supplementary Fig. 1).

Gene ontology analysis was next performed for those genes identified as being differentially expressed in the five distinct FA treatment groups. **Figure 1** shows an enrichment of genes in multiple gene ontology categories that would be expected to influence myocardial biology. These include genes regulating transcription, signaling, cell survival, and metabolism. In the case of palmitate and stearate, genes influencing apoptosis were highly represented; in particular, these saturated FAs induced a number of genes involved in ER and oxidative stress (**Table 1**). In contrast, FA/lipid metabolism was particularly enriched for both oleate- and linoleate-challenged ARCs. **Table 2** shows that not only does oleate induce a larger number of these genes (relative to other FA species), it generally induces these genes to a greater extent. For example, *ucp3* is induced 8.8-fold by oleate, but is not differentially expressed following challenge with palmitate. However, examples in which metabolic genes are differentially expressed following palmitate but not oleate challenge also exist, including *adrp* (induced 9.5-fold by palmitate, with no effect by oleate).

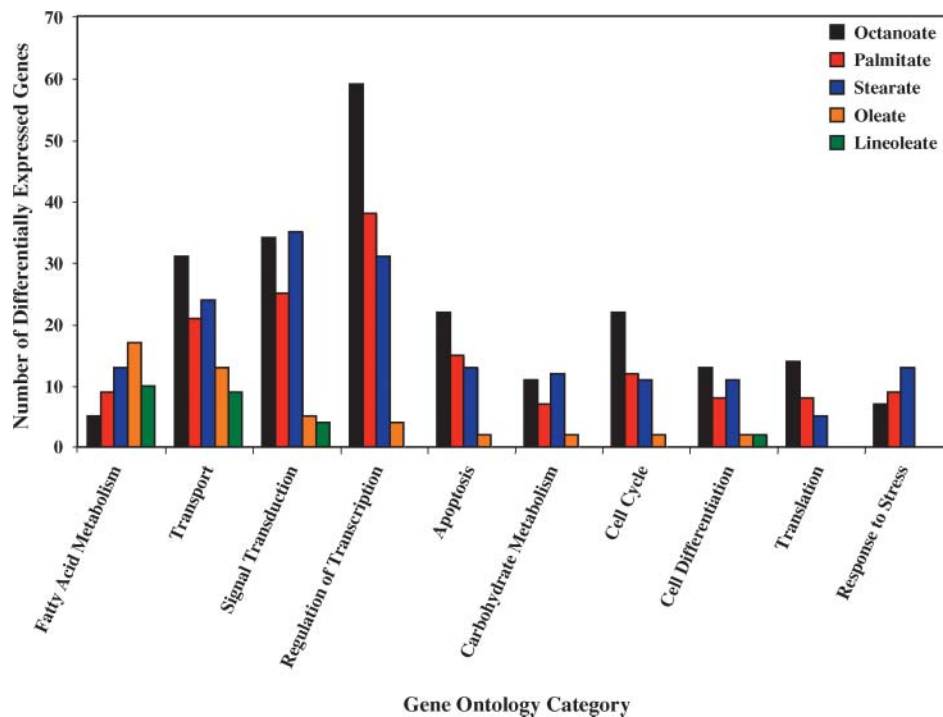


Fig. 1. Gene ontology analysis of differentially expressed genes 24 h after challenging adult rat cardiomyocytes (ARCs) with octanoate, palmitate, stearate, oleate, or linoleate. Data are presented as number of differentially expressed genes.

TABLE 1. Differential effects of distinct fatty acids on ARC expression of genes influencing apoptosis, cell survival, and stress response

Process	Gene	Protein	Octanoate	Palmitate	Stearate	Oleate	Linoleate
ER stress-induced apoptosis	<i>atf3</i>	Activating transcription factor 3 (ATF-3)	7.3↑	6.5↑	6.1↑	NS	NS
	<i>ddit3</i>	DNA damage-inducible transcript 3	NS	2.7↑	2.6↑	NS	NS
	<i>herpud1</i>	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein	1.8↑	2.5↑	1.9↑	NS	NS
	<i>hspa5</i>	Heat-shock 70 kDa protein-5	4.1↑	3.0↑	NS	NS	NS
	<i>phlda1</i>	Pleckstrin homology-like domain, family A, member 1	NS	5.9↑	5.8↑	NS	NS
Induction of apoptosis	<i>xbp1</i>	X-box binding protein 1	2.4↑	2.2↑	NS	NS	NS
	<i>bcl10</i>	B-cell CLL/lymphoma 10	2.8↑	2.1↑	NS	NS	NS
	<i>cidea_predicted</i>	Cell death-inducing DFFA-like effector a	NS	NS	NS	2.1↑	2.2↑
	<i>nme3</i>	Non-metastatic cells 3, protein expressed in	2.5↓	1.6↓	NS	NS	NS
	<i>pycard</i>	PYD and CARD domain containing	5.8↓	5.7↓	3.2↓	2.2↓	NS
	<i>siah2</i>	Seven in absentia homolog 2	2.6↑	2.8↑	2.3↑	NS	NS
	<i>stk17b</i>	Serine/threonine kinase 17b	4.4↑	5.1↑	5.3↑	NS	NS
	<i>tde1</i>	Tumor differentially expressed 1	NS	1.3↑	1.3↑	NS	NS
	<i>tieg</i>	TGFB inducible early growth response	NS	2.7↑	2.7↑	2.6↑	NS
	<i>trp53inp1</i>	Tumor protein p53-inducible nuclear protein 1	2.2↑	NS	1.7↑	NS	NS
Anti-apoptosis	<i>angptl4</i>	Angiopoietin-like 4	NS	12.8↑	NS	18.9↑	15.6↑
	<i>atf5</i>	Activating transcription factor 5	NS	2.8↑	NS	NS	NS
	<i>bdnf</i>	Brain-derived neurotrophic factor	2.7↑	3.2↑	NS	NS	NS
	<i>cfl1</i>	Cofilin 1 (non-muscle)	1.8↑	1.8↑	NS	NS	NS
	<i>dnajb9</i>	DnaJ (Hsp40) homolog, subfamily B, member 9	7.8↑	7.7↑	NS	NS	NS
	<i>gclc</i>	Glutamate-cysteine ligase, catalytic subunit	NS	3.8↑	NS	NS	NS
	<i>gnrh1</i>	Gonadotrophin-releasing hormone 1	3.1↑	2.6↑	NS	NS	NS
	<i>msh2</i>	MutS homolog 2	1.8↓	1.8↓	NS	NS	NS
	<i>prkdc_predicted</i>	Protein kinase, DNA-activated, catalytic polypeptide	3.0↓	2.5↓	2.2↓	NS	NS
	<i>prnp</i>	Prion protein (p27-30)	3.1↑	2.2↑	2.1↑	NS	NS
	<i>rtkn</i>	Rhotekin	1.8↓	2.0↓	2.8↓	NS	NS
	<i>sgk</i>	Serum/glucocorticoid regulated kinase	6.0↑	5.1↑	6.9↑	NS	NS
	<i>tra1_predicted</i>	Transformation/transcription domain-associated protein	3.2↑	2.7↑	NS	NS	NS
	<i>tpt1</i>	Tumor protein, translationally-controlled 1	NS	NS	1.8↑	NS	NS
	<i>txndc5_predicted</i>	Thioredoxin domain containing 5	NS	2.1↑	NS	NS	NS
Response to oxidative stress and DNA damage	<i>gadd45a</i>	Growth arrest and DNA-damage-inducible, α	2.6↑	4.0↑	4.2↑	NS	NS
	<i>gadd45b</i>	Growth arrest and DNA-damage-inducible, β	NS	2.1↑	3.0↑	NS	NS
	<i>gss</i>	Glutathione synthetase	NS	1.8↑	NS	NS	NS
	<i>hao1</i>	Hydroxyacid oxidase	NS	NS	3.0↑	NS	NS
	<i>hmox2</i>	Heme oxygenase (decycling) 2	NS	NS	1.5↑	NS	NS
	<i>txnrd1</i>	Thioredoxin reductase 1	NS	2.8↑	NS	NS	NS

ARC, adult rat cardiomyocyte; ER, endoplasmic reticulum; NS, nonsignificant.

Real-time RT-PCR was next employed to validate a number of genes identified as being differentially expressed in ARCs following challenge with distinct FA species. Seven genes were chosen: *ucp3* (promotes mitochondrial FA export), *mcd* (promotes mitochondrial FA import), *pdck4* (represses carbohydrate oxidation), *cte1* (promotes fatty acyl-CoA hydrolysis), *mte1* (promotes fatty acyl-CoA hydro-

lysis), *adrbp* (lipid droplet binding protein), and *phlda1* (ER stress marker). **Figure 2** compares the differential effects of distinct FAs on these genes, as assessed by microarray versus quantitative RT-PCR analysis. Similar to our microarray data, we find that two of the five metabolic genes (i.e., *ucp3* and *mcd*) were induced to the greatest extent by oleate, compared with the other FAs (Fig. 2A,

TABLE 2. Differential effects of distinct fatty acids on ARC expression of genes influencing fatty acid metabolism

Process	Gene	Protein	Octanoate	Palmitate	Stearate	Oleate	Linoleate
FA uptake	slc27a1	Fatty acid transport protein 1 (FATP1)	NS	NS	NS	3.7↑	3.4↑
	cd36	Fatty acid translocase (FAT)	NS	NS	NS	2.3↑	NS
	LOC499984	Similar to FAT	NS	1.9↑	NS	2.1↑	NS
	LOC499985	Similar to FAT	NS	NS	NS	2.5↑	2.3↑
Mitochondrial β -oxidation	acs11	Long-chain acyl-CoA synthetase 1 (ACSL1)	NS	2.9↑	NS	2.6↑	NS
	cpt1a	Carnitine palmitoyltransferase 1A (CPT1A)	NS	NS	NS	4.3↑	NS
	cpt1b	Carnitine palmitoyltransferase 1B (CPT1B)	NS	1.8↑	NS	2.0↑	2.1↑
	cpt2	Carnitine palmitoyltransferase 2 (CPT2)	1.8↓	NS	NS	1.6↑	NS
	mlycd	Malonyl-CoA decarboxylase (MCD)	NS	1.9↑	2.0↑	2.2↑	2.1↑
	slc25a20	Carnitine/acylcarnitine translocase (CACT)	NS	1.9↑	1.6↑	2.5↑	2.1↑
	slc25a29	Carnitine/acylcarnitine translocase-like (CACL)	NS	2.0↓	NS	1.8↓	1.9↓
	acad9	Very long chain acyl-CoA dehydrogenase (VLCAD)	1.9↓	1.5↓	NS	NS	NS
	crat	Carnitine acetyltransferase (CAT)	NS	2.1↑	1.8↑	2.3↑	2.2↑
	etfdh	Electron-transferring flavoprotein dehydrogenase (ETFDH)	NS	2.2↑	2.1↑	2.3↑	2.2↑
Triglyceride metabolism	hadha	Hydroxyacyl-CoA dehydrogenase, α subunit (HADH α)	NS	NS	1.4↑	1.5↑	NS
	hadhb	Hydroxyacyl-CoA dehydrogenase, β subunit (HADH β)	NS	1.6↑	1.8↑	1.6↑	1.6↑
	decr1	2,4-Dienoyl-CoA reductase 1 (DECRI)	NS	2.2↑	2.0↑	2.6↑	2.3↑
	gpam	Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM)	NS	NS	NS	3.2↑	NS
	lipe	Hormone-sensitive lipase (HSL)	NS	8.7↑	6.2↑	8.3↑	6.6↑
	aqp7	Aquaporin 7 (AQP7)	NS	7.1↑	6.0↑	9.6↑	7.2↑
	LOC361676	Adipose triglyceride lipase (ATGL)	NS	3.3↑	3.0↑	4.1↑	3.2↑
	LOC501283	Lipid storage droplet protein 5 (LSDP5)	NS	NS	NS	12.0↑	7.3↑
	adrp	Adipose differentiation-related protein (ADRP)	NS	9.5↑	10.7↑	NS	NS
	Phospholipid metabolism	chkb	Choline kinase β (CHK β)	NS	2.3↑	2.0↑	2.5↑
impa2		Inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2)	NS	1.8↑	NS	2.2↑	1.8↑
pik3c2g		Phosphoinositide-3-kinase, class 2, γ polypeptide (PIK3C2 γ)	NS	18.6↑	8.2↑	19.7↑	12.3↑
pik4cb		Phosphoinositol 4-kinase, catalytic, β polypeptide (PIK4C β)	NS	2.3↑	NS	2.0↑	1.9↑
ppap2b		Phosphatidic acid phosphatase type 2B (PPAP2B)	NS	2.2↑	NS	2.4↑	NS
Peroxisomal metabolism		acox1	Acyl-CoA oxidase 1 (ACOX1)	NS	NS	NS	1.9↑
	ech1	Enoyl-CoA hydratase 1, peroxisomal (ECH1)	NS	2.3↑	1.8↑	2.8↑	2.3↑
	hsd17b4	Hydroxysteroid (17 β) dehydrogenase 4 (HSD17 β 4)	1.7↓	NS	NS	1.4↑	1.4↑
	LOC315973	Acyl-CoA dehydrogenase family member 11 (ACAD11)	NS	NS	NS	2.0↑	NS
	nudt7_predicted peci	Peroxisomal CoA diphosphatase (NUDT7) Peroxisomal D3,D2-enoyl-CoA isomerase (PECI)	NS NS	6.0↑ 2.5↑	4.3↑ 2.5↑	5.7↑ 2.6↑	4.3↑ 2.2↑
FA-mediated futile cycling	bach	Acyl-CoA thioesterase 7 (ACOT7)	NS	3.5↑	3.4↑	3.8↑	3.2↑
	cte1	Cytosolic thioesterase 1 (CTE1)	NS	83.4↑	47.8↑	56.3↑	NS
	mte1	Mitochondrial thioesterase 1 (MTE1)	NS	20.4↑	NS	14.7↑	NS
	ucp2	Uncoupling protein 2 (UCP2)	NS	2.8↑	2.9↑	3.6↑	2.8↑
	ucp3	Uncoupling protein 3 (UCP3)	NS	NS	4.9↑	8.8↑	NS
Other	grcc3f_predicted	Membrane-bound O-acyltransferase domain containing 5 (MBOAT5)	NS	2.7↑	2.6↑	2.3↑	2.2↑
	pdk4	Pyruvate dehydrogenase kinase 4 (PDK4)	NS	23.1↑	34.4↑	28.1↑	NS

FA, fatty acid.

B, respectively). Stearate and oleate induced *pdk4* to a similar extent (Fig. 2C), whereas *cte1* and *mte1* were induced to the greatest extent by palmitate (Fig. 2D, E, respectively). Finally, palmitate and stearate induced *adrp* and *phlda1* to the greatest extent (Fig. 2F, G, respectively).

Time- and concentration-dependent effects of distinct FAs on metabolic gene expression

Given the large number of metabolic genes identified by our microarray studies as being differentially regulated by

distinct FA species, we decided to investigate the dynamics by which specific FA species induce genes. Exposure of cardiomyocytes to a FA at a single concentration, for a single duration, may provide only limited information regarding the response of the cardiomyocyte to that FA. For example, it is possible that palmitate and stearate rapidly induce expression of FAO-promoting genes (e.g., *ucp3*) at lower concentrations, whereas higher concentrations (such as 0.4 mM) activate counterregulatory mechanisms. We therefore investigated the time- and

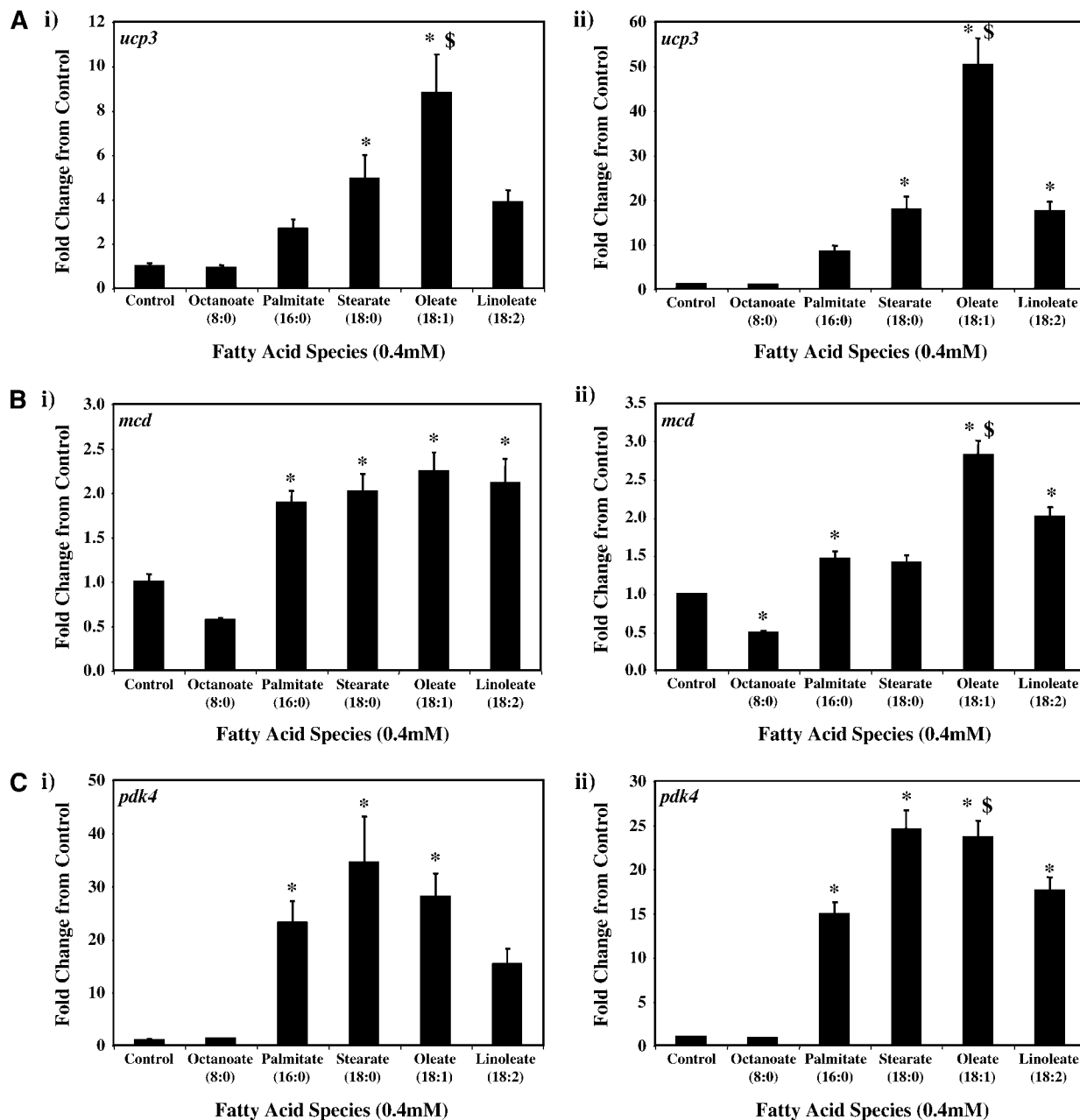


Fig. 2. RT-PCR validation of microarray analysis. Effects of distinct fatty acid (FA) species on ARC expression of *ucp3* (A), *mcd* (B), *pdk4* (C), *cte1* (D), *mtel* (E), *adrp* (F), and *phlda1* (G), as assessed by microarray analysis (i) and real-time RT-PCR (ii). All FA species were conjugated to 1% BSA, and were present at a concentration of 0.4 mM. Control cells were cultured in the absence of FA, but in the presence of 1% BSA. ARCs were challenged for 24 h. Individual gene expression data were initially normalized to the housekeeping gene *cyclophilin* and are presented as values relative to the control group. Data are shown as mean \pm SEM for between 7 and 8 observations (microarray analysis) or 21 observations (RT-PCR) per group. * $P < 0.05$, control versus specific FA; \$ $P < 0.05$, oleate versus palmitate.

concentration-dependent effects of distinct FAs on the selected genes previously utilized for microarray validation purposes. Here, ARCs were challenged with various concentrations of the investigated FAs (i.e., 0 mM, 0.1 mM, 0.2 mM, 0.4 mM) for multiple durations (i.e., 0, 3, 6, 12, 24, 48 h). For the purposes of clarity, **Fig. 3** reports the acute (6 h) and chronic (24 h) concentration-dependent effects of the distinct FAs. Data for the entire time course are presented in supplementary Figs. II–VIII.

Figure 3A shows that with the exception of octanoate, the FAs investigated induced *ucp3* expression in a concentration-

dependent manner. Acutely (i.e., 6 h), palmitate, stearate, and oleate induced *ucp3* to a similar extent. However, induction of *ucp3* was transient in nature for palmitate and stearate, whereas the effects of oleate persisted chronically (i.e., 24 h). A similar pattern was observed for *mcd* (Fig. 3B), although the level of induction of this gene was markedly lower, compared with that of *ucp3*.

As for *ucp3*, *pdk4* was rapidly induced by all long-chain FAs investigated, in a concentration-dependent manner (Fig. 3C). However, stearate acutely induced *pdk4* to the greatest extent, whereas chronically, stearate and oleate

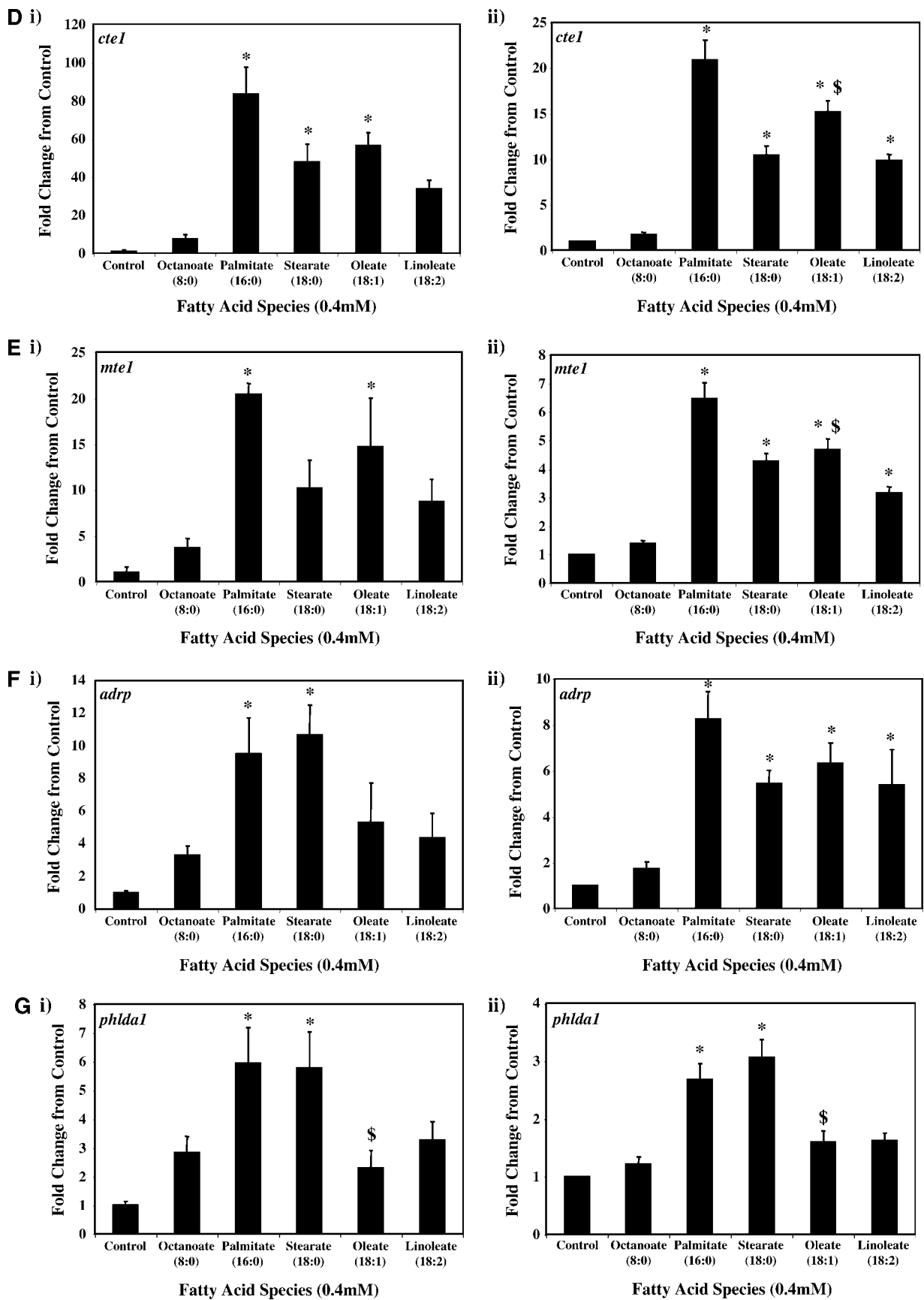


Fig. 2.—Continued.

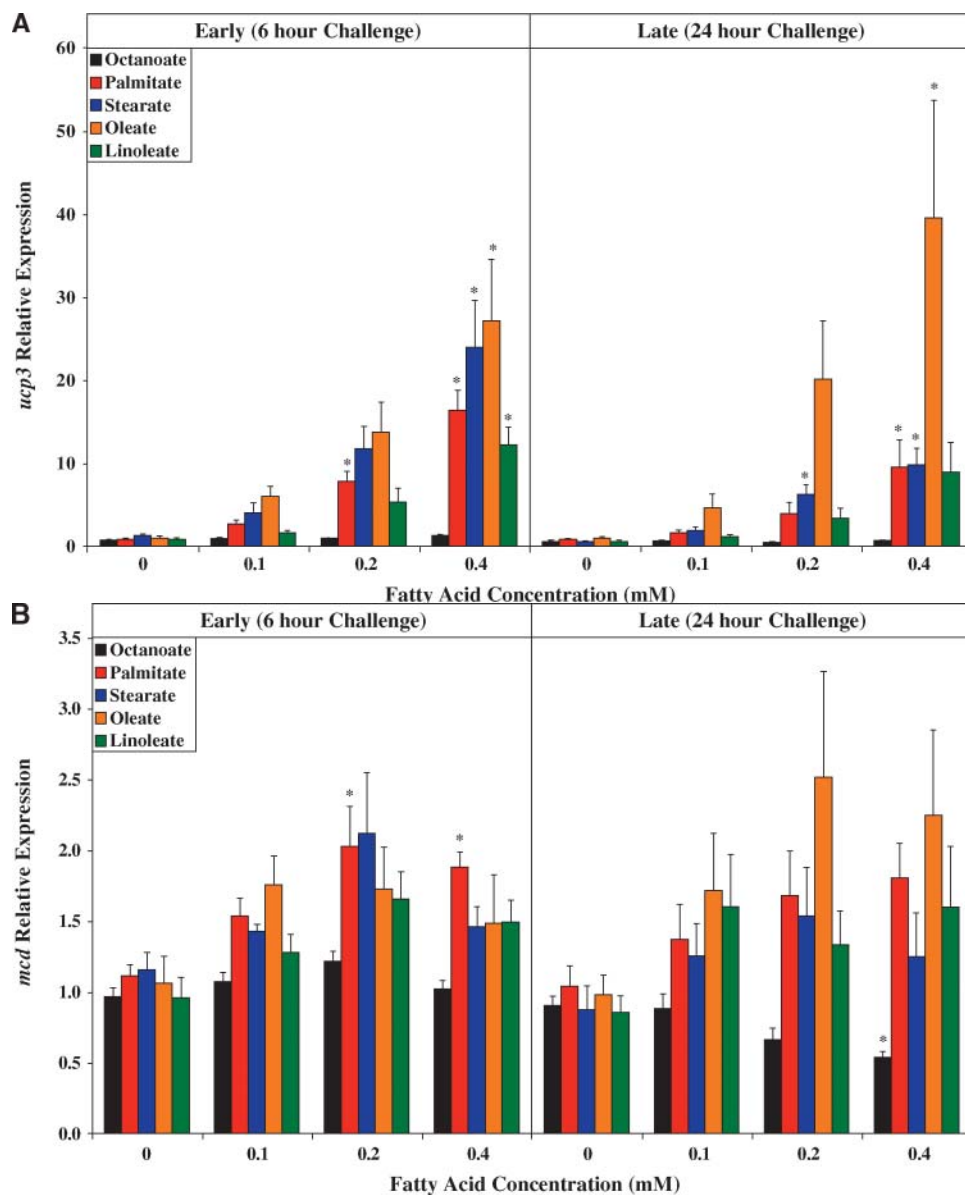


Fig. 3. Time- and concentration-dependent effects of distinct FA species on ARC expression of *ucp3* (A), *mcd* (B), *pdh4* (C), *cte1* (D), *mte1* (E), *adrp* (F), and *phlda1* (G). All FA species were conjugated to 1% BSA, and were present at a concentration of 0.1 mM, 0.2 mM, or 0.4 mM. Control cells were cultured in the absence of FA, but in the presence of 1% BSA. ARCs were challenged with FAs either acutely (6 h) or chronically (24 h). Individual gene expression data were initially normalized to the housekeeping gene *cyclophilin* and are presented as values relative to the control group (i.e., time zero). Data are shown as mean \pm SEM for between 4 and 5 observations. * $P < 0.05$, control (i.e., zero concentration) versus respective FA at distinct concentration.

had similar effects. Figure 3D, E shows that expression of *cte1* and *mte1* is influenced by distinct FAs in a similar manner to one another. Acutely, these thioesterases are induced by stearate to the greatest extent, whereas chronically, induction is greatest with palmitate. Virtually identical profiles were observed with *adrp* (Fig. 3F) and *phlda1* (Fig. 3G).

DISCUSSION

The primary goal of the present study was to investigate whether FAs of distinct carbon chain length and degree

of saturation elicit differential influences on ARC gene expression. We report that: 1) microarray profiling reveals that long-chain unsaturated FAs (e.g., oleate) induce a larger number of genes promoting FA oxidation compared with long-chain saturated FAs (such as palmitate); 2) palmitate and stearate induced markers of ER stress, as well as oxidative and DNA damage; 3) palmitate and stearate tended to induce FA-responsive metabolic genes only transiently; and 4) the short-chain FA octanoate influenced expression of a greater number of genes, relative to long-chain FAs. These observations reveal differential effects of distinct FAs on the transcriptional response of

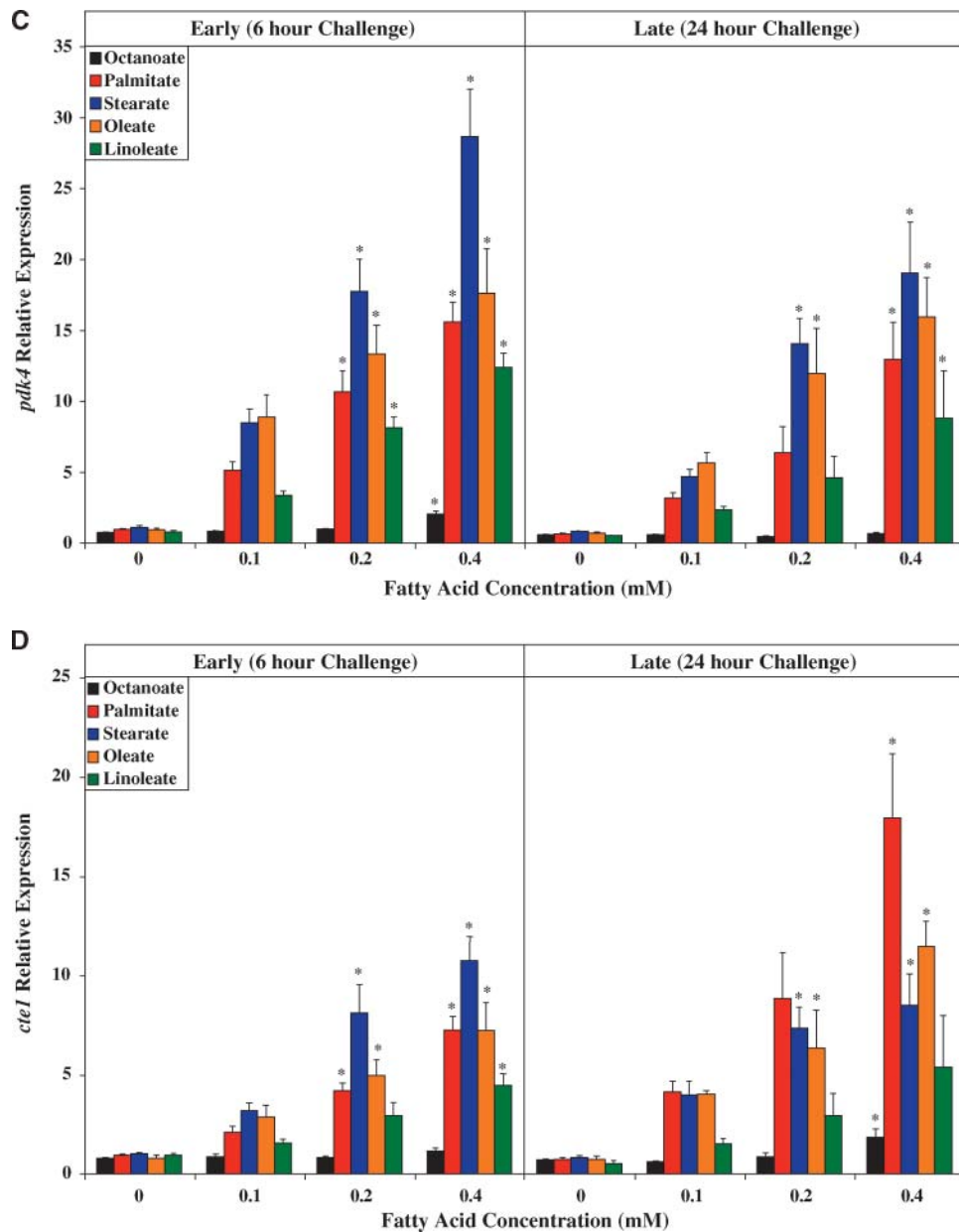


Fig. 3.—Continued.

ARCs, and may ultimately aid in elucidation of the mechanisms contributing to the cardioprotective versus the cardiotoxic effects of FAs on the heart.

The cardioprotective FA oleate induces genes promoting FA oxidation

Distinct FA species have divergent functions in the heart. For example, long-chain saturated FAs (e.g., palmitate and stearate) have been shown to induce apoptosis in cardiomyocytes, whereas oleate (a monounsaturated long-chain FA) rescues cardiomyocytes from palmitate-induced lipotoxicity (19, 20). Fatty acid composition is altered by diet, resulting in altered expression of proteins that regulate FA metabolism, which, in turn, may influence the ability of the heart to avoid lipotoxic damage. We recently observed that feeding rats a diet rich in unsaturated FAs re-

duced cardiac ceramide content and the frequency of cardiomyocyte apoptosis compared with a high-saturated-fat diet (28). In addition, high-fat diets composed of either medium- or long-chain saturated FAs, or long-chain unsaturated FAs, differentially induced the expression of known PPAR α -regulated genes (28, 29). However, one must use caution in drawing conclusions about the effects of chain length and saturation from in vivo feeding studies, because such studies do not allow for assessment of the direct effects of individual FAs. At present, the direct effects that various saturated and unsaturated FAs have on myocardial gene expression are poorly understood.

Through a comprehensive, unbiased transcriptional profiling (i.e., microarray analysis) we report that oleate (as well as linoleate) influenced the expression of a relatively small number of genes (83 for oleate), compared

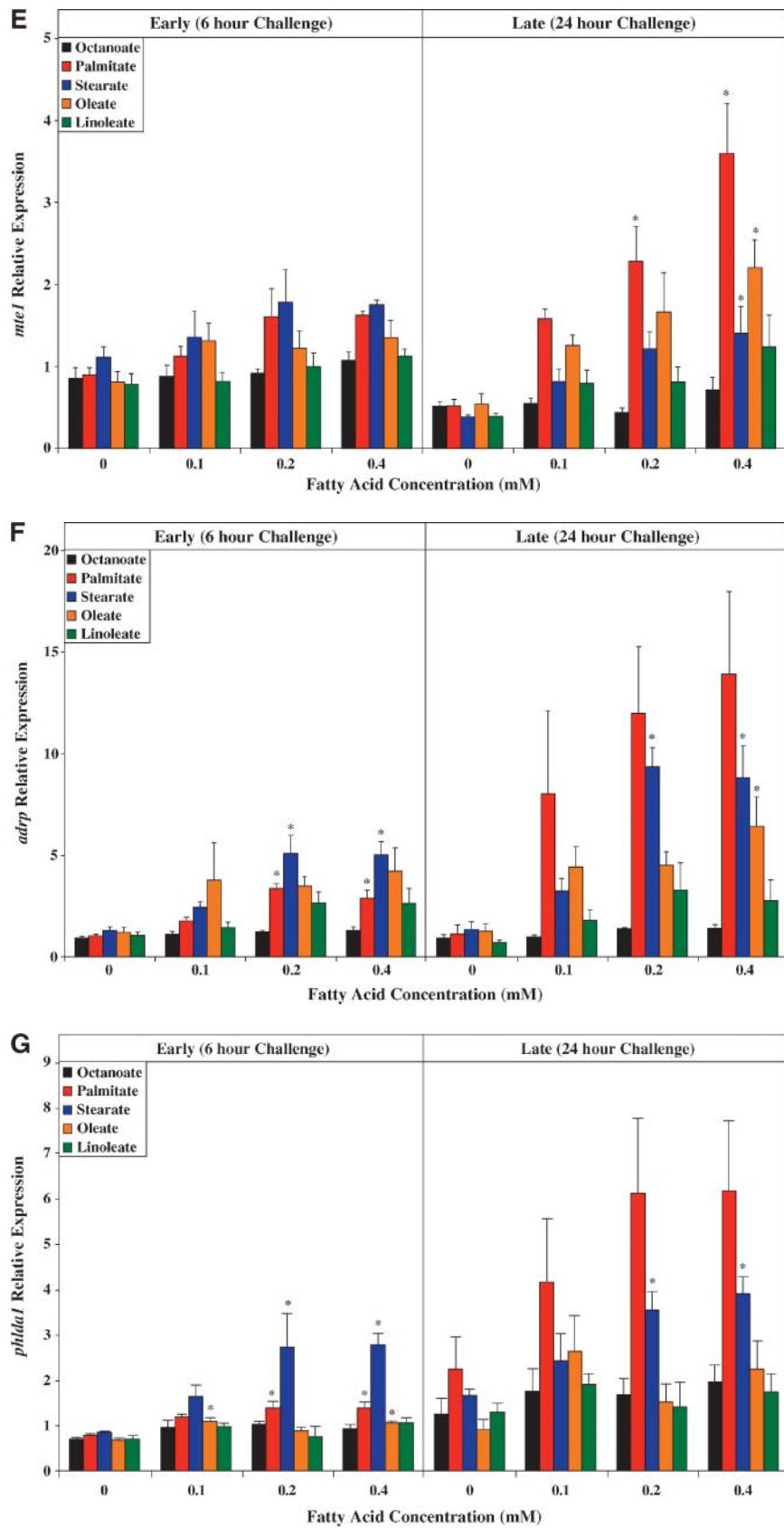


Fig. 3.—Continued.

with octanoate, palmitate, and stearate, of which 75% were induced (see supplementary Table III). Furthermore, of these genes, a large proportion are known to promote FAO (Fig. 1). When comparisons are made between the cardioprotective FA oleate and the cardiotoxic FA palmitate, for genes promoting FA metabolism, several interesting observations are made. First, a larger number of these metabolic genes are induced by oleate compared with palmitate (Table 2). Furthermore, when both of these FAs induce the same gene, the effect is generally larger for oleate. These generalizations are particularly noticeable for genes involved in long-chain FA uptake and mitochondrial β -oxidation. In the case of *mcd* and *ucp3* (both believed to promote FA oxidation), the greater induction by oleate was confirmed by RT-PCR (Fig. 2A, B).

A number of specific differentially expressed genes are worthy of additional discussion. MTE1 and UCP3 have been proposed to work in tandem, in which MTE1 hydrolyzes FA-CoAs to free FAs, which are subsequently exported out of the mitochondrial matrix by UCP3 (30). In doing so, MTE1/UCP3 probably promotes FA oxidation by preventing the sequestration of free CoA required for continued β -oxidation. However, a relatively high induction of *mte1* by palmitate (Fig. 2C), in the absence of induction of *ucp3* (Fig. 2E), would promote accumulation of free fatty acyl groups within the mitochondrial matrix (as a long-chain acyl-CoA synthetase has not been identified within the mitochondrial matrix). Accumulation of palmitate within the mitochondrial matrix would probably promote apoptosis. Another notable difference in the transcriptional effects of oleate versus palmitate is in regard to lipid storage droplet proteins (LSDPs). LSDPs bind to lipid droplets, thereby influencing the latter's stability and metabolic fate (31). Five LSDPs have been identified to date, of which ADRP and LSDP5 are highly expressed in the heart (32–34). Microarray analysis revealed that oleate induces *ldsp5* gene expression to a greater extent than palmitate, whereas palmitate induces *adrp* gene expression to a greater extent than oleate (Table 2). Interestingly, ER-derived ADRP-coated lipid droplets have been shown to specifically activate xanthine oxidase in mammary gland epithelial cells, resulting in reactive oxygen species (ROS) production (31, 35). Given the strong supportive evidence that palmitate-induced lipotoxicity involves ROS production (36), and that xanthine oxidase inhibition has been shown to be cardioprotective (37), an attractive hypothesis regarding palmitate-induced apoptosis is that ADRP-rich lipid droplets induce ROS production through xanthine oxidase activation following palmitate exposure. Given that ADRP-rich lipid droplets are synthesized in the ER, and that ADRP-rich lipid droplets are enriched with saturated (palmitate and stearate) versus unsaturated (oleate) long-chain FAs (35), the possibility arises that this mechanism may contribute to palmitate-induced ER stress. A greater induction of LSDP5, in response to oleate, may attenuate ADRP-rich lipid droplet formation, thereby protecting the cardiomyocyte.

The cardiotoxic FA palmitate induces markers of ER and oxidative stress

When exposed to elevated levels of specific long-chain saturated FAs (e.g., palmitate, stearate) for prolonged periods of time (e.g., 24–48 h), certain cell types (e.g., pancreatic β -cells, cardiomyocytes) initiate apoptosis (19, 20, 36, 38, 39). Initial studies investigating the potential molecular mechanisms for this lipotoxicity have focused largely on mitochondrially based processes, such as inhibition of the electron transport chain (by ceramide, for example), ROS production, diminution of the inner-mitochondrial membrane proton motive force, and cytochrome c release (36, 40, 41). More recently, potential extra-mitochondrial mechanisms have been explored. Borradaile et al. (38) have recently provided evidence that palmitate-induced apoptosis in HeLa/COS cells is potentially mediated through the ER stress response. Furthermore, pharmacological stimulation of FAO reduced palmitate-induced ER stress and apoptosis, presumably because of increased oxidation of this lipotoxic FA (38).

Our microarray analysis revealed that the long-chain saturated FAs palmitate and stearate induced a markedly larger number of genes in ARCs, compared with oleate and linoleate (Table 1). We reasoned that genes known to impact apoptosis, whose expression is differentially influenced by palmitate/stearate versus oleate/linoleate, may provide vital clues to the mechanisms by which long-chain saturated FAs induce lipotoxicity. Following this logic, two genes known to promote ER stress-induced apoptosis (*ddit3* and *phlda1*) were induced exclusively by palmitate and stearate; these observations were confirmed by RT-PCR for *phlda1*. Additional markers of ER stress (e.g., *atf3* and *herpud1*) were also induced by both palmitate and stearate (although these genes were also induced by the medium-chain FA, octanoate). These observations are consistent with those of Borradaile et al. (38), which suggest that palmitate induces ER stress in HeLa/COS cells.

Previously published studies suggest that palmitate-mediated ROS generation in cardiomyocytes probably plays a role in palmitate-induced apoptosis (36). Consistent with this possibility, our microarray analysis reveals that exposure of ARCs to 0.4 mM palmitate for 24 h leads to an induction of multiple markers of oxidative stress and DNA damage (Table 1). These include *gadd45a/b*, *gss*, and *txnrd1*. Similar observations are seen with ARCs exposed to stearate, but not following exposure to oleate or linoleate (Table 1).

Acute versus chronic effects of FAs on ARC gene expression

An obvious question relates to whether alterations in FAO gene expression initiate apoptosis, whether initiation of apoptosis causes alterations in FAO gene expression, or whether these events are mutually exclusive. A time course study was therefore performed, to compare early (acute) changes versus late (chronic) changes in an attempt to provide potential insight regarding this issue. When comparisons are made between oleate and palmitate, we find

that oleate induces *ucp3* to a greater extent than does palmitate, both acutely and chronically. Consistent with palmitate-induced apoptosis being a later event, palmitate induces the ER stress marker *phlda1* to a greater extent than does oleate only chronically. These data support the hypothesis that oleate induces cardioprotective genes, such as *ucp3*, which help protect against apoptosis. Consistent with this concept, McLeod et al. (42) have shown that *ucp3* is cardioprotective during ischemia/reperfusion. Clearly, future studies are required to definitely determine whether promotion of FAO is indeed the mechanism by which oleate protects the myocardium from palmitate-induced apoptosis.

Identification of novel potential mechanisms for FA-induced cardiotoxicity/cardioprotection

Microarray analysis and subsequent gene ontology revealed differential effects of distinct FAs on cardiomyocyte processes (e.g., FA metabolism, apoptosis), as discussed above. However, several additional candidate genes for cardiotoxicity/cardioprotection were identified by our microarray analysis, several of which warrant further discussion. Given the opposing effects of palmitate/stearate versus oleate on cardiomyocyte survival, we reasoned that genes differentially expressed between palmitate- and oleate-challenged cells, as well as between stearate- and oleate-challenged cells, may reveal novel potential mechanisms involved in FA-induced cardiotoxicity/cardioprotection. One example is *rgs7bp* (regulator of G-protein signaling 7 binding protein; LOC499521). Expression of this gene is 553-fold higher in stearate-challenged cells, and 372-fold higher in palmitate-challenged cells, versus oleate-challenged cells; this is due to induction by both palmitate and stearate, as well as a slight repression by oleate (relative to control cells; see supplementary Table II). RGS7BP has been shown to modulate G protein signaling in the brain, although this function has not been investigated previously in the heart (43). Interestingly, RGS7BP requires reversible palmitoylation to anchor the protein at the cell surface, where it modulates G protein signaling; removal of palmitate from culture media causes relocation of RGS7BP to the nucleus (43). Thus, challenging cardiomyocytes with palmitate would cause not only a dramatic induction of RGS7BP, but also anchoring of the protein to the cell surface. That RGS7BP-mediated alterations in cardiomyocyte signaling contribute to cardiotoxicity is a novel and testable hypothesis. This analytical approach also identified genes that were more highly expressed in oleate-challenged cardiomyocytes, relative to palmitate- and stearate-challenged cells (such as *ucp3*; see above discussion). These genes might be considered as candidates for cardioprotection. One example is *mitocalcin* (LOC501181). Expression of this gene is 14.8- and 6.9-fold higher in oleate-challenged cells relative to palmitate- and stearate-challenged cells, respectively (see supplementary Table II). MITOCALCIN is a novel mitochondrial Ca^{2+} binding protein with EF-hand and coiled-coil domains (44). Although no known function has been elucidated for this protein, knockdown of the gene in 2Y3t cells re-

sults in cell death (44). One could therefore hypothesize that repression by palmitate and stearate contributes to apoptosis, perhaps through impairing mitochondrial Ca^{2+} handling.

Medium-chain FAs induced marked alterations in ARC gene expression


One surprising observation in the current study is that challenging ARCs with the medium-chain FA octanoate induced the largest number of changes in myocardial gene expression, as compared with the long-chain FAs investigated (see supplementary Table II). A total of 1,188 genes were differentially expressed in octanoate-exposed versus control ARCs. These observations are consistent with previously published studies showing that medium-chain FAs induce alterations in gene expression within other cell types, such as hepatocytes and adipocytes (45–47). Clearly, the current study design is unable to delineate the precise mechanism(s) by which this occurs. However, one possible hypothesis arises upon inspection of the gene ontology analysis (Fig. 1). Although octanoate influences expression of the largest number of total genes, this FA has little effect on FA metabolism genes (presumably because of an inability to activate PPAR α and/or PPAR β/δ). Palmitate influences expression of a larger number of genes involved in FA metabolism compared with octanoate, whereas the total number of differentially expressed genes decreases. In contrast, oleate influences expression of the greatest number of FA metabolism genes, whereas the total number of differentially expressed genes is very low. These observations led us to speculate that insufficient activation of pathways involved in FA utilization, when availability increases (such as the case with octanoate), is “sensed” by the cardiomyocyte, resulting in substantial alterations in gene expression. Given the large number of genes influenced, it could be hypothesized that a central gene expression mechanism might be involved. One such candidate is acetylation; increased carbon availability, in the absence of full oxidation/utilization, would provide acetyl groups for acetylation reactions. Indeed, studies in yeast have shown that acetylation-mediated alterations in gene expression are driven primarily through acetyl-CoA availability (48). If the hypothesis is correct, simple consumption of diets rich in medium-chain FAs (i.e., feeding studies) would probably not result in significant alterations in myocardial gene expression, in large part because of the energy demands of the beating heart (as compared with quiescent cells in culture).

Study limitations, perspectives, and future directions

The primary limitations of the current study include its descriptive nature and focus on alterations in gene expression. Conclusions regarding either the precise mechanisms by which distinct FAs differentially influence cardiomyocyte gene expression, or whether these alterations in gene expression manifest concomitant alterations in protein expression and activity cannot be made. As such, our observations are limited to generation of

novel hypotheses that remain to be interrogated. Previous studies have shown that unsaturated long-chain FAs are more potent ligands for the PPARs, as compared with saturated FAs, consistent with the idea that PPAR α and PPAR β/δ mediate the induction of genes promoting FA oxidation (49, 50). However, the mechanisms responsible for the greater number of genes influenced by palmitate, stearate, and octanoate remain unidentified. One cannot rule out the possibility that the transient nature in which palmitate and stearate influence ARC metabolic gene expression is secondary to initiation of apoptosis and/or acceleration of dedifferentiation. However, the current study is the first to investigate the effects of distinct FA species on adult cardiomyocytes. The majority of previously published studies have focused on the effects of a specific FA species on candidate gene expression in neonatal cardiomyocytes (16, 17). Given the prominent alterations in myocardial nuclear receptors and metabolic substrate switching during the first 3 weeks of life for the rodent, the present study provides an improved understanding of the direct effects of distinct FA species on the adult heart.

SUMMARY

The present study profiles the transcriptional response of ARCs to distinct FAs. We report that long-chain saturated FAs influence ARC gene expression in a manner that is quite distinct from that of long-chain unsaturated FAs. Specifically, the long-chain unsaturated FAs oleate and linoleate influenced the expression of a relatively small subset of genes, within which many genes promoting FA oxidation were induced. In contrast, the long-chain saturated FAs palmitate and stearate influence expression of a larger number of genes, yet fewer are known to promote FA oxidation. Furthermore, a greater subset of genes involved in ER and oxidative stress were induced by palmitate. These observations may ultimately aid in the elucidation of the mechanisms contributing to the cardioprotective versus the cardiotoxic effects of FAs on the heart. 

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