Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes

Karin A. J. M. van der Lee, Michaël M. Vork, Johan E. De Vries, Peter H. M. Willemsen, Jan F. C. Glatz, Robert S. Reneman, Ger J. Van der Vusse, and Marc Van Bilsen[1]

Department of Physiology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands

Abstract Long-chain fatty acids are the most important substrates for the heart. In addition, they have been shown to affect signalling pathways and gene expression. To explore the effects of long-chain fatty acids on cardiac gene expression, neonatal rat ventricular myocytes were cultured for 48 h with either glucose (10 mM), fatty acids (palmitic and oleic acid, 0.25 mM each), or a combination of both as exogenous substrates. Exposure to fatty acids (both in the absence or presence of glucose) neither affected cellular morphology and protein content nor induced alterations in the expression of phenotypic marker genes like atrial natriuretic factor and the Ca-ATPase SERCA2. However, incubation with fatty acids (with or without glucose) resulted in up to 4-fold increases of the mRNA levels of fatty acid translocase (FAT/CD36), heart-type fatty acid-binding protein, acyl-CoA synthetase, and long-chain acyl-CoA dehydrogenase. In contrast, the expression of genes coding for proteins involved in glucose uptake and metabolism, i.e., glucose transporter GLUT4, hexokinase II, and glyceraldehyde 3-phosphate dehydrogenase, remained constant or even declined under these conditions. These changes corresponded with a 60% increase in cardiomyocyte fatty acid oxidation capacity. Interestingly, the peroxisome proliferator-activated receptor–α (PPARα)–ligand Wy 14,643, but not the PPARγ–ligand ciglitazone, also resulted in increased mRNA levels of genes involved in fatty acid metabolism. In conclusion, fatty acids specifically and co-ordinately up-regulate transcription of genes coding for proteins involved in cardiac fatty acid transport and metabolism, most likely through activation of PPARα.—van der Lee, K. A. J. M., M. M. Vork, J. E. De Vries, P. H. M. Willemsen, J. F. C. Glatz, R. S. Reneman, G. J. Van der Vusse, and M. Van Bilsen. **Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myoctyes.** *J. Lipid Res.* **2000.** 41: **41–47.**

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Cardiac function is dependent on a continuous supply of such nutrients as long-chain fatty acids and glucose (1). Recent findings indicate that, in addition to being an important energy source, fatty acids are also involved in signal transduction pathways in various tissues including the heart (2). In preadipocytes, exposure to long-chain fatty acids was found to markedly affect gene expression and differentiation (3, 4). Likewise, the administration of fatty acids to C2C12N skeletal myoblasts inhibited their differentiation into myotubes and actually induced the expression of adipocyte-specific genes, like aP2, the adipocyte lipid-binding protein (5).

As for now, the effects of fatty acids on gene expression in the cardiac myocyte are only beginning to be appreciated. In a preliminary study we showed that in neonatal cardiac myocytes fatty acids are able to increase the mRNA level of heart-type fatty acid-binding protein (H-FABP) (6). Using the same model system, it was demonstrated recently that fatty acids enhanced the transcriptional activity of the muscle-type carnitine palmitoyltransferase I (M-CPTI) gene (7). These observations prompted us to investigate in more detail the effects of long-chain fatty acids on cardiomyocyte phenotype in general and on the expression of genes encoding for proteins involved in glucose and fatty acid metabolism in particular. Primary cultures of neonatal rat ventricular cardiomyocytes were chosen as the model system, as the supply of substrates can be easily manipulated (8). In the present study the cardiomyocytes were incubated in minimal medium to which either glucose, fatty acids (palmitic acid and oleic acid complexed to albumin), or a combination of glucose and fatty acids had been added. The effects of these substrates on cellular phenotype were assessed cytochemically, by measuring cellular protein content, and through monitoring the expression of phenotypic markers, like atrial natriuretic factor (ANF) and cardiac Ca^{2+}–ATPase (SERCA2). The effects of substrate supply on the mRNA levels of a range of proteins involved in glucose handling, i.e., glucose transporter type 4 (GLUT4), hexokinase II (HexII), and glyc-

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Abbreviations: ANF, atrial natriuretic factor; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FAT, fatty acid translocase; H-FABP, cardiac fatty acid-binding protein; ACS, acyl-CoA synthetase; LCAD, long-chain acyl-CoA dehydrogenase; GLUT4, glucose transporter type 4; HexII, hexokinase II.

[1] To whom correspondence should be addressed.

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eraldehyde 3-phosphate dehydrogenase (GAPDH), and various stages of fatty acid uptake and metabolism, i.e., fatty acid translocase (FAT/CD36), cardiac fatty acid-binding protein (H-FABP), acyl-CoA synthetase (ACS), and long-chain acyl-CoA dehydrogenase (LCAD) were evaluated. Because mRNA levels of proteins related to fatty acid uptake and metabolism were found to be up-regulated after exposure to fatty acids, the functional consequence of this up-regulation for the cellular capacity to oxidize fatty acids was also investigated. Finally, as fatty acids have been implicated as natural ligands for transcription factors of the family of peroxisome proliferator-activated receptors (PPARs) (9, 10), the effects of specific PPAR–ligands were evaluated.

MATERIALS AND METHODS

Cell culture

Primary cultures of neonatal rat ventricular myocytes were prepared as described previously (8). Experiments were approved by the Institutional Animal Care and User Committee of the Maastricht University. Cardiomyocytes were plated at low density (250 cells/mm^2) in tissue culture dishes coated with 1% gelatin type B (G-9382, Sigma, St. Louis, MO) in a 4:1 mixture of DMEM (GIBCO-42430, GIBCO-BRL Life Technologies, Gaithersburg MD) and M199 (GIBCO 31153) supplemented with 10% horse serum (GIBCO 16050), 5% newborn calf serum (Sera-Lab, Sussex, UK), and antibiotics (P/S; penicillin 100 IU/ml and streptomycin 0.1 mg/ml, GIBCO). Overnight incubation in serum-rich medium was followed by 24 h incubation in serum-free medium of a 4:1 mixture of DMEM/M199, containing P/S and 10 mm glucose as the sole substrate. Subsequently, the myocytes were rinsed once with substrate-free medium (4:1 mixture of DMEM (GIBCO 11963) and glucose-free M199 (GIBCO 31153)), to which l-carnitine (0.25 mm), insulin (0.25 mU/ml), and bovine serum albumin (BSA 0.15 mm; Sigma A-7906) had been added. This medium was supplemented with different (combinations of) substrates. The first experimental group received only glucose (final concentration 10 mm) as substrate. The second group received a mixture of palmitic acid (C16:0) and oleic acid (C18:1) (0.25 mm each; complexed to 0.15 mm BSA as described elsewhere (8)). The third group received a combination of both substrates, i.e., glucose (10 mm) and C16:0/C18:1 (0.25 mm each). After 48 h of incubation, cardiomyocytes were either harvested for RNA isolation, used for cytochemical staining, or for measuring the rate of fatty acid oxidation.

In a separate series of experiments, cells receiving only glucose (10 mm) as substrate, were incubated with 100 μm Wy 14,643 (Biomol, Plymouth Meeting PA) or 10 μm ciglitazone (Biomol), specific ligands for PPARα and PPARγ, respectively. Stock solutions of Wy 14,643 and ciglitazone were prepared in DMSO. After 48 h of incubation cardiomyocytes were harvested for RNA isolation.

Analysis of mRNA expression

Total RNA was isolated with TRIzol reagent (GIBCO). RNA (5 μg) was size-fractionated on a denaturating gel (1% agarose, 6.6% formaldehyde, 1 $\times$ MOPS), transferred to a nylon membrane (Hybond-N, Amersham, Slough, UK) by capillary transfer, and fixed using standard techniques. After prehybridization, the filters were probed with a 1600 bp XbaI–XhoI fragment of rat GLUT4 cDNA (a gift from Dr. D. James, University of Queensland, Australia), a 1390 bp EcoRI fragment of rat LCAD (a gift from Dr. K. Tanaka, Yale University, USA), a 2500 bp EcoRI fragment of rat FAT (kindly provided by Dr. N. A. Abumrad, State University New York, USA), a 676 bp EcoRI-BamHI fragment of H-FABP, a 700 bp HindIII-BamHI fragment of ANF (gift from Dr. K. Chien, University of California San Diego, USA), a 320 bp NsiI-EcoRI fragment of SERCA2 (a gift from Dr. W. Simonides, Free University Amsterdam, The Netherlands), a 520 bp EcoRV-HindIII fragment of ACS (gift from Dr. T. Yamamoto, Tohoku University, Sendai, Japan), a 3200 bp EcoRI-KpnI fragment from HexII (gift from Dr. E. Wilson, Michigan State University, USA) and a 1400 bp EcoRI-HindIII fragment from GAPDH. The cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) by random-priming (Radprime, Life Technologies) to a specific activity of $>0.5 \times 10^9$ cpm/μg DNA. To correct for possible differences in transfer and loading the filters were also hybridized with a ^{32}P-labeled ribosomal 18S probe. After hybridization, filters were washed at the appropriate stringency to remove non-specific binding. The filters were exposed to phosphor imaging screens and subsequently scanned with a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA), and quantified using ImageQuant software (Molecular Dynamics).

Cytochemistry

After fixation of the cells in alcoholic formalin (90% ethanol, 4% formalin in doubly distilled H_2O) at room temperature for 1 min, glycogen was visualized by periodic acid-Schiff (PAS) staining (11). Neutral lipids (triacylglycerols) were visualized with Oil Red-O (Aldrich Chemical Company, Milwaukee, WI) as described elsewhere (8). PAS- and Oil Red-O-stained cells were counterstained with hematoxylin (Sigma GHS-2-16).

Fatty acid oxidation

In a subset of experiments, fatty acid oxidation capacity was determined of cardiomyocytes after 48 h of incubation in the presence of the different substrates. Oxidation experiments were performed as modified from Luiken et al. (12), using a buffer solution containing (in mm), HEPES (20), NaH_2PO_4 (1), $MgSO_4$ (0.4), $CaCl_2$(1), NaCl (120), KCl (5), BSA (0.3), glucose (5), palmitic acid (0.05), oleic acid (0.05), pH 7.4. After preparation the buffer was vigorously stirred in order to ensure full oxygen saturation. Attached cells (10 cm culture dishes) were pre-incubated in buffer (5 ml) for 30 min at 37°C. Subsequently, a trace amount of [1-^{14}C]palmitic acid was added and the pre-incubation was continued for an additional 30 min in order to reach a steady-state situation. Thereafter, a vial containing 500 μl CO_2-trapping medium (NaOH, 0.1 m) was inserted and the dish was subsequently sealed airtight. Oxidation was terminated immediately (t = 0) or after 30 min (t = 30) by injection of 500 μl $HClO_4$ (5 m) through a silicone seal on the lid of the dish. Pilot studies had shown that CO_2 production was linear for at least 60 min (data not shown). Dishes were stored at 4°C overnight after which trapping medium was assessed for ^{14}C activity by liquid scintillation counting. Oxidation capacity was calculated by the trapped ^{14}C activity at t = 30 minus trapped ^{14}C activity at t = 0 and was expressed as nmol $CO_2 \cdot min^{-1} \cdot mg^{-1}$ total protein. Total protein was quantified using the micro BCA method (Pierce, Rockford, IL) with BSA as standard.

Statistics

Results are obtained from at least six different cell isolations and presented as means ± SD. Comparison between groups was performed with one-way analysis of variance (ANOVA). When the F-ratio obtained indicated that significant differences between groups were present, a two-tailed Student's *t*-test for unpaired data was carried out, applying Bonferroni's adjustment for multiple comparison (13). Differences were considered significant at $P < 0.05$.

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RESULTS

Fatty acids and cardiomyocyte phenotype

First, it was investigated whether the phenotype of neonatal ventricular myocytes was affected by the addition of fatty acids. After 48 h of culture in defined medium containing either glucose (10 mm), fatty acids (0.25 mm C16:0 and 0.25 mm C18:1), or a combination of glucose plus fatty acids as substrate, differences in cellular morphology were not apparent (e.g. no differences in sarcomere content or organization). In all groups the cells were irregular in shape and of comparable size. Differences in cellular protein content were not observed (**Table 1**). These observations indicate that the mere presence of C16:0/C18:1 in the culture medium for 48 h does not give rise to obvious phenotypical alterations of the cardiomyocytes. The latter finding is corroborated by the fact that fatty acids neither induced expression of the hypertrophic marker gene atrial natriuretic factor (ANF) nor affected SERCA2 mRNA levels (Table 1).

Endogenous substrate stores

Despite the absence of overt differences in cardiomyocyte phenotype, cytochemical staining revealed that variation of exogenous substrate supply induced marked changes in intracellular substrate stores (**Fig. 1**). When glucose was the only substrate available, neutral lipids were occasionally observed and appeared as small red droplets in the cytoplasm (Fig. 1A). In contrast, glycogen was abundantly present throughout the cytoplasm (Fig. 1B). Exposing the cells to fatty acids instead of glucose resulted in the depletion of glycogen granules and a marked increase of lipid droplets (Figs. 1C and 1D). In the presence of glucose as well as fatty acids, the cardiomyocytes accumulated substantial amounts of both glycogen and triacylglycerols during the 48-h incubation period (Figs. 1E and 1F).

Expression of genes involved in metabolism

Next it was tested whether the changes in substrate handling, as suggested by the cytochemical observations, were associated with changes in the expression of genes involved in glucose and fatty acid transport and metabolism. Northern blot analysis of neonatal myocytes cultured in the presence of different (combinations of) substrates revealed that the mRNA levels of FAT, H-FABP, ACS, and LCAD were strongly affected by the presence of C16:0/C18:1 in the culture medium (**Fig. 2**). Relative to cardiomyocytes exposed to glucose as the sole substrate, there was a 2- to 4-fold increase in the mRNA levels of FAT, H-FABP, ACS, and LCAD when cells received either fatty acids as the sole substrate, or a combination of glucose and fatty acids (Fig 2A). In contrast, when fatty acids were present in the medium either as the sole substrate or in combination with glucose, the mRNA levels of GLUT4 significantly declined to 56% and 41%, respectively, of the level in cells receiving glucose as the only substrate (Fig. 2B). The changes of HexII and GAPDH were in the same direction, but less pronounced.

TABLE 1. Effect of substrate on cardiomyocyte protein content and ANF and SERCA2 expression

Substrate	Protein Content	ANF mRNA	SERCA2 mRNA
Glucose	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.4
Fatty acids	0.8 ± 0.1	1.1 ± 0.7	0.8 ± 0.2
Glucose + fatty acids	0.9 ± 0.2	0.8 ± 0.7	1.0 ± 0.2

Differences in total cellular protein content or ANF and SERCA2 mRNA levels were not detected. The levels relative to the control group (only glucose as substrate), the level of expression of which was arbitrarily set at 1.0, are shown. Messenger RNA levels of ANF and SERCA2 were first normalized to the corresponding 18S ribosomal RNA signal to correct for possible differences in loading. Data presented as means ± SD of at least 6 independent cell cultures.

Fatty acid oxidation

The rate of fatty acid oxidation was determined in intact cells after 48 h of incubation with glucose only, fatty acids only, or the combination of glucose and fatty acids. The oxidation of exogenously administered [1-^{14}C]palmitate, as measured as $^{14}CO_2$ production, amounted to 0.12 ± 0.04 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein in cardiomyocytes previously exposed to glucose as the only substrate. When the cells had received fatty acids as substrate (with or without glucose), an almost 70% increase in the rate of $^{14}CO_2$ production was observed (**Fig. 3**). This finding indicates that the fatty acid-mediated activation of genes involved in fatty acid transport and metabolism is accompanied by an enhanced catabolic flux through this metabolic pathway.

Effects of PPAR–ligands

As fatty acids have been implicated to act as natural ligands for PPARs (14), it was investigated whether Wy 14,643 (100 μm) and ciglitazone (10 μm), established ligands for PPARα and PPARγ, respectively, were able to mimic the effects of fatty acids on cardiomyocyte gene expression. At these concentrations, Wy 14,643 and ciglitazone were previously shown to be effective and isoform-specific in other cell types (15, 16). In cells receiving glucose as the sole substrate, the addition of the PPARγ-specific ligand ciglitazone did not have any effect on the mRNA levels of ACS, LCAD, or GLUT4 (**Fig. 4**). In contrast, administration of the PPARα-selective ligand Wy 14,643 led to markedly enhanced mRNA levels of ACS and LCAD, without affecting GLUT4 mRNA levels.

DISCUSSION

It is demonstrated for the first time that exposure of neonatal rat ventricular myocytes to long-chain fatty acids enhances their capacity to oxidize fatty acids by selectively and co-ordinately increasing the mRNA levels of genes involved in cellular fatty acid transport, i.e., FAT/CD36 (17–19) and H-FABP (20, 21), as well as fatty acid metabolism, i.e., ACS and LCAD.

In the present study, palmitic (C16:0) and oleic acids (C18:1) were chosen as exogenous fatty acid substrates as, from a quantitative point of view, these fatty acids constitute

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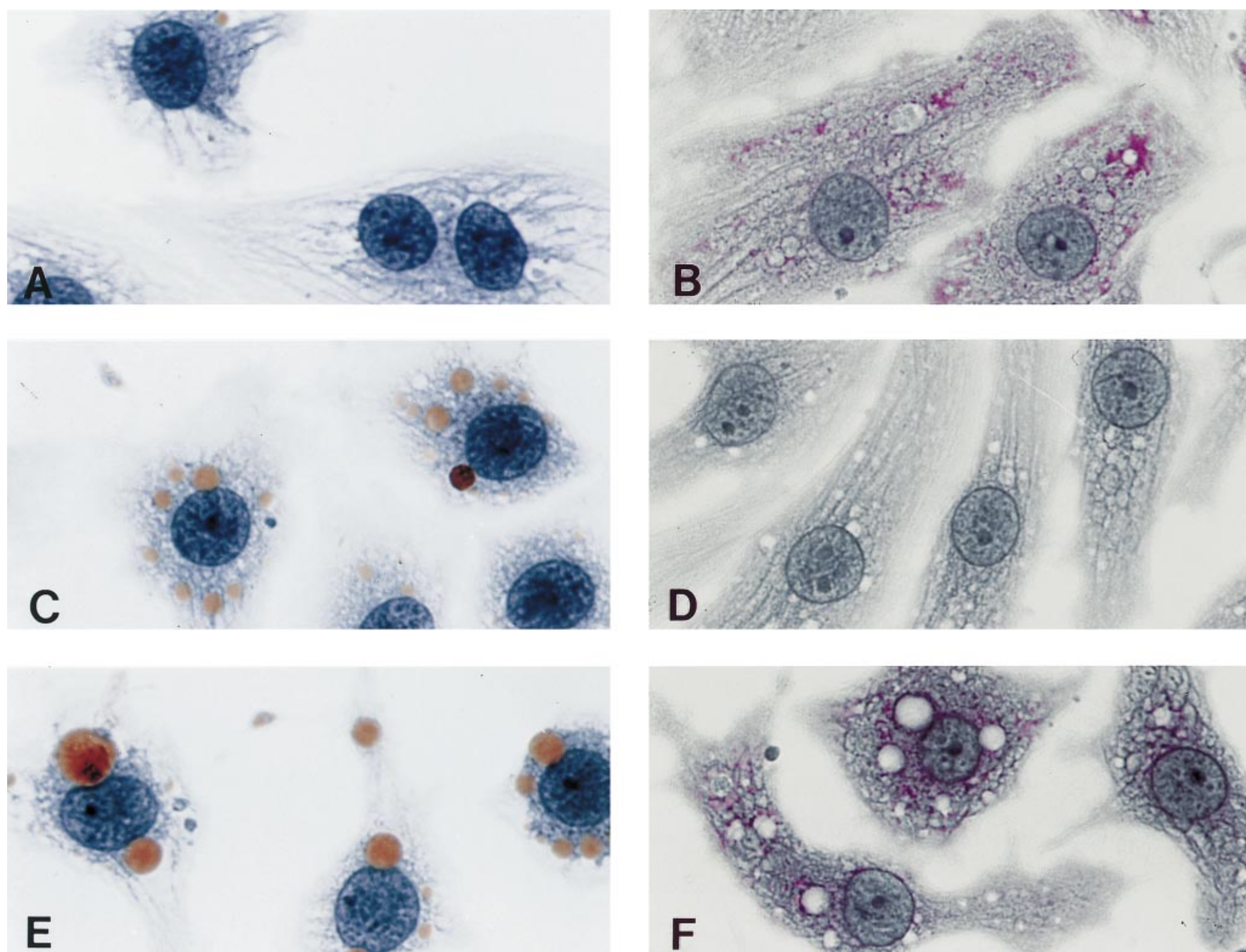

Fig. 1. Effect of substrate on triacylglycerol (panels A,C,E) and glycogen (panels B,D,F) content in neonatal cardiomyocytes. To visualize neutral lipids, cells were stained with Oil Red-O. The periodic acid–Schiff reagent was used to detect glycogen (pink staining). Cells were cultured in the presence of either glucose (A,B), fatty acids, i.e., palmitate/oleate mixture (C,D), or a combination of glucose and these fatty acids (E,F) for 48 h.

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the most important fatty-acyl moieties in rat blood (22). Previous findings (8) (M. M. Vork, unpublished observations) indicated that these fatty acids are readily taken up, incorporated in endogenous lipid pools, and oxidized by the neonatal myocytes. As opposed to polyunsaturated fatty acids, C16:0 and C18:1 do not function as precursors of eicosanoids. This implies that the observed effects on gene expression are due to the fatty acids themselves, rather than to the formation of biologically active cyclooxygenase or lipoxygenase products, which have been shown to act as auto/paracrine factors inducing cardiomyocyte hypertrophy (23). The observation that exposure to saturated and monounsaturated fatty acids neither gives rise to changes in cellular protein content nor to the induction of hypertrophic marker genes like ANF supports this notion.

Along with the marked increases in the mRNA levels of genes involved in fatty acid handling, the mRNA content of proteins involved in glucose handling (GLUT4, HexII, GAPDH) tended to decline. Generally speaking, however, the changes observed in the mRNA levels appear to be less consistent and less pronounced. The physiological relevance of these changes, as far as glucose metabolism is concerned, remains to be established.

Putative mechanism of fatty acid-mediated gene expression

Effects of fatty acids on gene expression have also been documented in studies with other cell types, such as adipocytes, hepatocytes, and skeletal muscle cells (4, 5, 24). As far as the mechanism is concerned, evidence is accumulating for the existence of fatty acid-mediated transcriptional regulation of various genes involved in lipid metabolism. In a number of cases the responsive *cis*-regulatory element has been identified as a peroxisome proliferator responsive element (PPRE) which binds peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor family (14, 25, 26). Recent studies have provided strong evidence that, in addition to prostaglandins, long-chain fatty acids may act as natural ligands for PPARs (10, 27). The PPARs display limited specificity with respect to various long-chain fatty acid species. Of note is the recent observation that the affinity of palmitic and oleic acids for

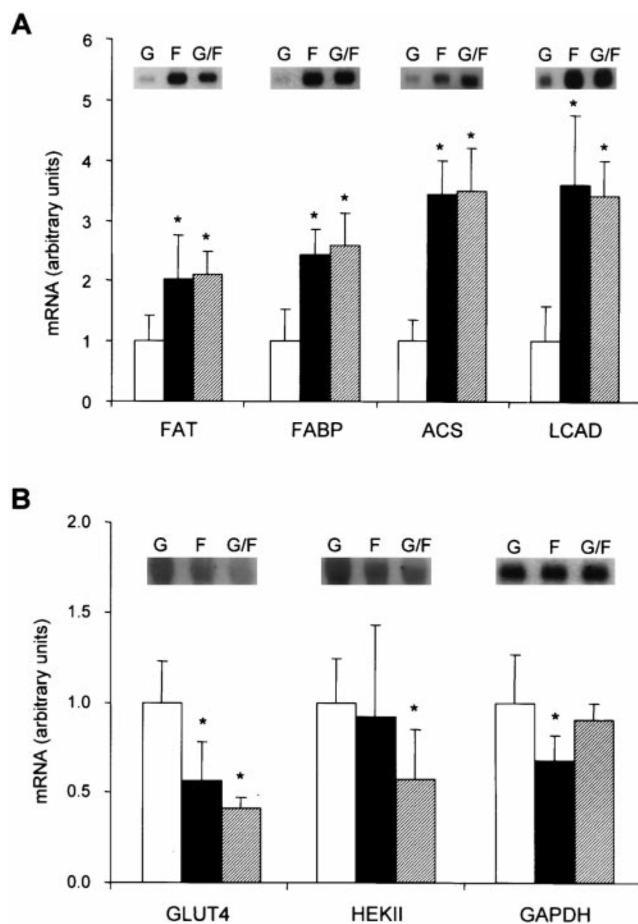

Fig. 2. Effect of fatty acids on cardiomyocyte mRNA levels. Cells were cultured with either glucose (G, open bars), fatty acids (F, black bars), or glucose plus fatty acids (G/F, hatched bars) for 48 h. Changes in mRNA levels of fatty acid translocase (FAT/CD36), fatty acid-binding protein (H-FABP), acyl-CoA synthetase (ACS), and long-chain acyl-CoA dehydrogenase (LCAD) are shown in panel A. Glucose transporter type 4 (GLUT4), hexokinase II (HexII), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in panel B. Representative Northern blot signals of the mRNAs investigated are included. The expression levels relative to the control group (only glucose as substrate), the level of expression of which was arbitrarily set at 1.0, are shown. mRNA levels were first normalized to the corresponding 18S ribosomal RNA signal to correct for differences in loading. Data are presented as means ± SD of at least 6 independent cell cultures; * indicates significantly different ($P < 0.05$) from the glucose group.

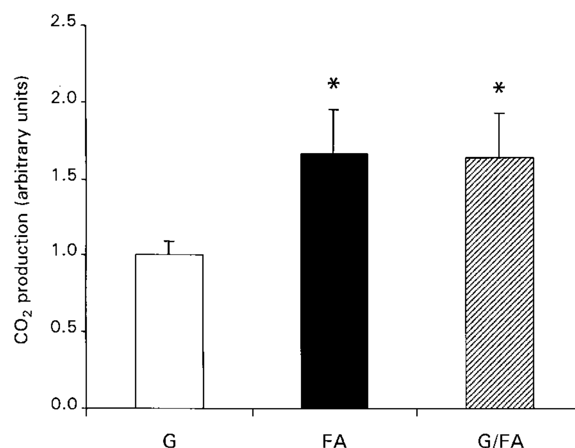

Fig. 3. Cardiomyocyte long-chain fatty acid oxidation capacity. Neonatal cardiomyocytes were cultured in the presence of either glucose (G), fatty acids (F), or glucose plus fatty acids (G/F) as substrate(s) for 48 h. Subsequently [1-^{14}C]palmitate oxidation was measured as the steady state rate of $^{14}CO_2$ production (the glucose group being arbitrarily set at 1.00). Data are presented as means ± SD of 5 independent cell cultures; * indicates significantly different ($P < 0.05$) from the glucose group.

the different PPAR isoforms is comparable to that of polyunsaturated fatty acids (28).

Both the α and β isoform, but probably not the γ isoform, of PPAR are expressed in the heart (see ref. 29 for review). The current finding that a PPARα ligand, but not a PPARγ ligand, is able to affect gene expression in cardiomyocytes supports the notion that PPARγ is either not present and/or of limited functional importance for cardiac myocytes. In line with this notion is the observation that the PPARγ ligand ciglitazone stimulates the expression of GLUT4 in adipocytes (30), whereas no such effect is observed in neonatal cardiomyocytes (see Fig. 4). The observation that FAT expression in liver is induced by PPARα ligands, but not by PPARγ ligands (31), also fits with this idea.

The fact that fatty acids and the PPARα-specific ligand Wy 14,643 have similar effects on cardiomyocyte mRNA levels supports the idea that these effects of fatty acids are PPARα-mediated. Indeed, a variety of genes are involved in cardiac lipid metabolism, among which FAT/CD36 and ACS are PPAR-responsive (14, 29, 31). Just recently it was demonstrated that the expression of muscle-type CPTI is also PPARα-dependent (32) and that exposure of neonatal cardiomyocytes to long-chain fatty acids activates transcription of the CPTI promoter via a PPRE (7).

Pathophysiological implications

Under normal conditions glucose and fatty acids each serve as substrates for the cardiac muscle. In adult rats fatty

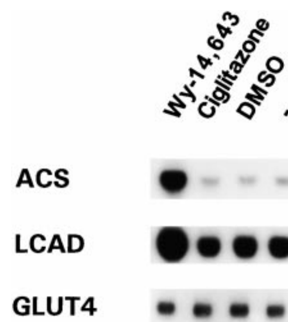

Fig. 4. Representative Northern blot, displaying the effect the PPARα ligand Wy 14,643 and the PPARγ ligand ciglitazone on cardiomyocyte mRNA levels of acyl-CoA synthetase (ACS), long-chain acyl-CoA dehydrogenase (LCAD), and glucose transporter type 4 (GLUT4). Cardiomyocytes were cultured in minimal medium, with glucose as the only substrate, without any addition (−), or in the presence of DMSO (vehicle), ciglitazone (10 μm) or Wy 14,643 (100 μm) for 48 h.

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acids are the substrate of preference, the oxidation of which covers over 60–70% of the cardiac energy requirements (1, 33). It is of interest to note that cardiac substrate preference undergoes substantial alterations during development. Carbohydrates are the substrates of choice for the fetal heart, whereas in the postnatal period the heart rapidly switches to fatty acids as its main energy source (34). It is generally believed that the alterations in substrate utilization and concordant changes in the activity of enzymes involved in fatty acid utilization are primarily caused by the postnatal changes in circulating levels of hormones, such as insulin, glucagon, and thyroid hormone (35). The present findings, however, suggest that in addition to hormonal regulation, the change in nutrient supply around birth (i.e., from maternal blood to lipid-rich milk) in the intact animal could be directly responsible for these in vivo changes.

Furthermore, several pathophysiological conditions (e.g., diabetes and cardiac hypertrophy and failure) are associated with a marked change in the plasma concentrations of substrates and/or a marked shift in substrate preference of the cardiac muscle cells (36–38). Evidence is accumulating that the metabolic adaptation most likely can be ascribed to alterations in the expression of the genes involved (39–41). It remains, however, to be determined whether fatty acid-induced and/or PPAR-mediated changes in cardiac gene expression contribute to the etiology of these diseases.

In conclusion, it is demonstrated that long-term exposure of cardiac muscle cells to fatty acids evokes a co-ordinated up-regulation of the expression of genes involved in lipid metabolism, with a concomitant increase in the cellular capacity to oxidize exogenous fatty acids. These findings clearly indicate that the cardiac myocyte is responsive to metabolic signals. In addition, evidence is provided that PPARs are likely to be part of the signalling pathway that conveys this metabolic signal to the transcriptional machinery of the cardiomyocyte.

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