Induction of cardiac FABP gene expression by long chain fatty acids in cultured rat muscle cells

Weihua Chang, Jutta Rickers-Haunerland and Norbert H. Haunerland
Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada

Abstract

The induction of cardiac FABP expression by long-chain fatty acids was measured in cultured rat myoblasts, myotubes and adult cardiomyocytes. With quantitative RT-PCR techniques, the primary transcription product of the FABP gene and the mature mRNA were measured. Incubations of 30 min resulted in a larger than 2-fold increase of the primary transcript in all cells, and FABP mRNA more than doubled in myoblasts and cardiomyocytes after 10 h of fatty acid exposure. The results demonstrate that long chain fatty acids induce the expression of the cardiac FABP gene in muscle cells and their undifferentiated precursors at the level of transcription initiation, suggesting that all factors involved in fatty acid dependent gene induction are already present in myoblasts. Thus, myoblast cell lines should be useful for the characterization of fatty acid response elements that control the expression of the FABP gene. (Mol Cell Biochem **221**: 127–132, 2001)

Introduction

Fatty acid-binding proteins (FABPs) are ubiquitous cytosolic proteins involved in fatty acid transport and metabolism [1]. Within the cytosol of cardiac and skeletal muscle cells, free fatty acids are bound by cardiac fatty acid-binding protein (H-FABP); this protein assures efficient transport of fatty acids towards mitochondrial beta-oxidation (for recent reviews, see [2, 3]). H-FABP is also believed to buffer the intracellular environment against the potentially damaging accumulation of unbound free fatty acids. The H-FABP content varies widely between different muscles and organisms but appears to be linked to the rate fatty acid utilization [4]. In various physiological experiments it has been demonstrated that increased fatty acid uptake and metabolism result in elevated concentrations of H-FABP and its mRNA, thus suggesting that fatty acids control the expression of the H-FABP gene [5–7]. However, the molecular mechanism of this induction remains unknown.

Fatty acid mediated induction of gene expression has also been observed for other members of the FABP gene family, and more detailed knowledge is available for adipocyte and liver FABP [8, 9]. Studies with mammalian liver and adipose cell lines have demonstrated that fatty acids induce directly the transcription rate of the respective FABP genes, and the *cis*-acting elements have been identified in reporter gene studies. Few experiments, however, have been carried out with cultured muscle cells. H-FABP is expressed in myoblast cell lines, namely mouse C2C12 cells [10] and rat L6 cells [11], but at much lower levels than in differentiated muscle cells. While the latter report could not find an induction of H-FABP expression upon palmitate exposure, systematic studies in this regard have not been carried out, and it remains unknown whether fatty acids also lead to the increased H-FABP production in myoblast cultures. In contrast, long-time incubations of neonatal cardiomyocytes clearly result in increased H-FABP mRNA levels [6, 12]. These experiments, however, did not reveal whether fatty acids directly influence the transcription of the H-FABP gene. Conclusive proof for this and knowledge of the mechanisms involved are experimentally difficult to obtain in differentiated muscles for which no stable cell lines exist. Transfections with reporter gene constructs have only rarely been carried out successfully in adult myocytes. While such experiments are easily performed in

myoblast cell lines, such as rat L6 cells, it is not known whether the regulation of H-FABP expression is comparable in these precursor cells, especially with respect to its induction by fatty acids.

The current study was carried out to investigate whether H-FABP expression in myoblasts is up-regulated by fatty acids in a similar manner as in differentiated muscle cells. The results strongly suggest that this up-regulation is initiated rapidly at the level of transcription initiation, and that myoblasts can be used as a valid model system to study the molecular elements involved in the regulation of the H-FABP gene.

Materials and methods

Preparation of heart tissue and cardiomyocytes

Male Sprague-Dawley rats were housed in the Animal Care facility of Simon Fraser University. Prior to heart dissection, rats were intra-peritoneally anesthetized with sodium pentobarbital. The heart was rapidly excised, placed into ice cold dissecting solution, and via the aorta attached to a water-jacketed Langendorff perfusion apparatus. Following perfusion first with aerated dissecting solution and then with collagenase solution, the digested tissue was gently disintegrated, and cells were transferred to culture dishes [13].

Cell culturing and isolation

Fresh cardiomyocytes were cultured at approximately 60–80% confluency in Delbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 150 μg/ml penicillin and 150 μg/ml streptomycin. L6 myoblasts were grown for 3 days to approximately 80% confluency. The medium was then replaced with medium that contained the specified concentration of the fatty acid/bovine serum albumin (BSA) complex, and incubated for the indicated time intervals. DMEM was used without FBS for incubations up to 6 h, while longer incubations times required the addition of 1% serum to the medium. Untreated controls were incubated under identical conditions, except that no fatty acid/BSA complex was added to the media. Prior to RNA extraction, cells were detached with 0.25% trypsin in phosphate buffered saline (0.01 M Phosphate, 0.9% NaCl, pH 7.4).

Myotubes were obtained from cultured myoblasts, similar as described [14]. Briefly, myoblasts were grown for 24 h to reach 20–30% confluency. At this time, cells were washed twice with DMEM and the medium was replaced with DMEM supplemented with 6% heat inactivated horse serum, and allowed to grow for a further 3–4 days. Short-term incubations were carried out in serum-free DMEM, while 1% inactivated horse serum was added for incubation times longer than 6 h.

Treatment with fatty acids

Fatty acid (600 μM)/BSA (200 μM) solutions were prepared by dissolving fatty acid free BSA (Sigma, Oakville, ON, Canada) and sodium salts of fatty acids (Sigma, Oakville, ON, Canada) in 0.9% NaCl. The fatty acid/BSA complex solutions were filtered sterile and added to the cell culture medium. In preliminary experiments, the final concentration of linoleic acid was varied between 60–240 μM. Since no significant differences in the increase of mRNA were observed at higher concentrations, the final concentrations of fatty acids used normally was 60 μm , unless otherwise indicated.

RNA isolation

RNA from cells was isolated using the ToTally-RNA kit from Ambion (Austin, TX, USA), without DNAse treatment. RNA was used immediately or stored in ethanol at –80°C for up to 4 weeks. The RNA was normally free of contaminating DNA, as confirmed by PCR with primers specific for exon 1 and 3 of the FABP gene (R1 and R4, see below). Samples containing any traces of DNA were discarded.

RT-PCR of primary transcript and mRNA

Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were brought to final volume of 50 μl and 500–800 ng of total RNA were added. Primers were added at a concentration of 0.2 nM. Primers specific for exon 1 (upper primer R1, TAG CAT GAC CAA GCC GAC CAC AAT C) and exon 3 (lower primer R4, GTT CCC GTG TAA GCT TAG TCT CCT G) were used to amplify a 224 bp fragment of H-FABP mRNA, and intron-specific primers that flank exon 2 (upper primer R12, GTT GCC AAC CTT CCC AGA CAT CCA C, lower primer R13, TCC CAG CAC TGA GCA GGC TTT ATG A) were used to yield a 493 bp fragment of the unspliced H-FABP primary transcript (Fig. 1). Following incubation for 10 min at 25°C, 10 min at 60°C, and 15 min at 42°C, the reverse transcription reaction was terminated by heating to 95°C for 5 min and cooled to 4°C. The mixture was denatured at 95°C for 1 min and amplified for 31 cycles of 30 sec at 95°C, 30 sec at 58°C, 1 min at 72°C. The reaction mixture was cooled down to 4°C. To exclude the presence of contaminating DNA, a control amplification with primers R1 and R 4, but without the reverse transcription reaction was carried out for each RNA sample used to determine H-FABP primary transcript. To determine the optimal conditions for quantitative analysis, the number of cycles was varied from 21 to 41. The PCR products increased in a linear way between 26 and 33 cycles when using mRNA primers, and between 28 and 32 cycles with the primary transcript primers.
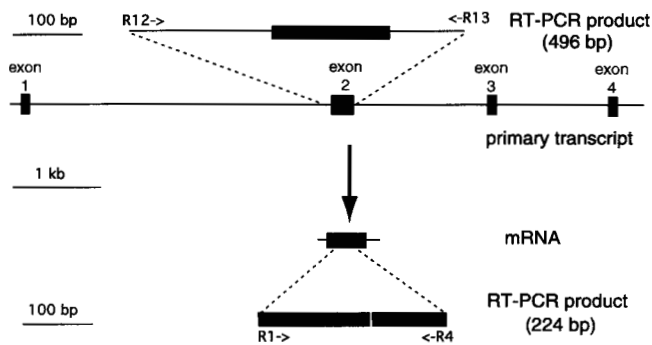
Fig. 1. PCR strategy. The primary transcript and the mRNA of the rat heart FABP gene (Genbank accession number AF144090) are shown, as well as the location of the PCR primers used in this study. For sequence information, see Materials and methods.

Quantification

Quantification of H-FABP RNA was achieved by multiplex PCR. The H-FABP PCR products were compared with those obtained with a set of primers specific for 18 S ribosomal RNA (product size = 324 bp). Because of the relative abundance of ribosomal RNA, inactive 18 S primers (competimers) were added to the active 18 S primers (QuantumRNA 18 S Internal Standard kit by Ambion, Austin, TX, USA). For the quantification of both mRNA and primary transcript in myoblasts and myotubes, the optimal ratio of 18 S specific primers:competimers was found to be 2:8, while samples isolated from primary cardiomyocytes required a lower competimer concentration (ratio 4:6 for mRNA, 3:7 for primary transcript). To determine the optimal template concentrations, between 5 and 2000 ng were used as template for the PCR reactions in preliminary experiments. Optimal results were obtained for 500–800 ng of RNA.

Results

Expression of H-FABP in L6 myoblasts, myotubes, and differentiated cardiomyocytes

In order to compare the expression of the H-FABP gene in undifferentiated and differentiated myocytes, both H-FABP mRNA and the primary transcript of the H-FABP gene were measured by reverse-transcription PCR, yielding PCR products of 224 bp and 493 bp, respectively (Fig. 2). To account for variations in template amount, 18 S RNA sequence was used as internal standard. A mixture of regular and blocked primers (competimers) was necessary to achieve similar intensities between the gene specific PCR products and the 324 bp 18 S PCR product. As shown in Fig. 2, H-FABP mRNA and the primary transcript can be found in myoblasts,

myotubes, and adult cardiomyoctes, with clear increases of both, primary transcript and mRNA with differentiation. Quantitative comparison with the PCR products from myoblasts indicate that the primary transcript levels in myotubes and cardiomyocytes are ~ 3 and 7 times as high, respectively. mRNA levels apparently increase more than 2-fold upon differentiation to myoblasts as well. Because of the relative abundance of H-FABP mRNA in the heart, 18 S RNA could only be seen when the concentration of the unblocked ribosomal RNA primers was increased (Fig. 2).

Conditions for stimulation with linoleic acid

Various treatment schemes were employed to find optimal conditions for fatty acid incubation. To include the possibility that fatty acid derived metabolites, e.g. eicosanoids, stimulate gene expression, linoleic acid was chosen for these experiments. Although fatty acid concentrations of up to 240 μM were tolerated by myoblasts in short-term incubations, longer exposure times of 10 h or more gave consistent results only for fatty acid concentrations below 120 μM. In preliminary experiments, similar increases in H-FABP expression were seen for smaller fatty acid concentrations, and hence all experiments were carried out in the presence of 60 μM fatty acids, complexed to fatty acid-free bovine serum albumin. Optimal, reproducible increases of primary transcript and mRNA were seen following incubations with fatty acid-containing media for 30 min or 10 h, respectively. H-FABP primary transcript increased within minutes after exposure of the cells to this fatty acid complex, and it remained high for several hours. In contrast, H-FABP mRNA increases were not seen for at least 4 h, and approached its maximal values only after 10 h (data not shown). Although mRNA levels frequently remained high for 24 h or more, cell viability decreased with longer incubation periods, making quantitative comparisons difficult.
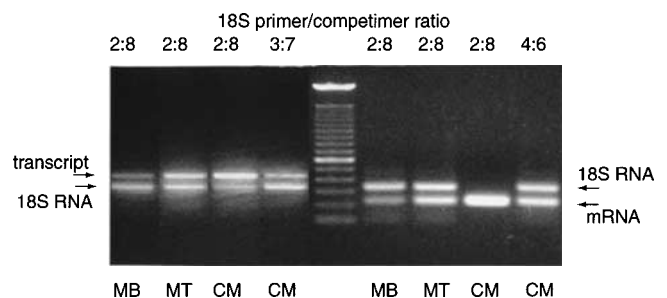

Fig. 2. H-FABP primary transcript and mRNA content in muscle cells. Left panel: pre-mRNA (amplified with primers R12 and R13); right panel: mRNA (amplified with primers R1 and R4). In all instances, 500 ng RNA were used as template, isolated from: MB – myoblasts; MT – myotubes; CM – cardiomyocytes. The specific primer:18 S competimer ratio is indicated above the lanes.

Stimulation by fatty acids

To study the induction of H-FABP expression by fatty acids, cultured myoblasts and myotubes, as well as isolated cardiomyocytes were treated with linoleic acid. As shown in Fig. 3, H-FABP primary transcript was clearly elevated in all cells after 30 min incubation, while the 18 S product remained constant. Similarly, mRNA increased in myoblasts and cardiomyocytes following 10 h of incubation (Fig. 3). Quantitative analysis revealed a 2–3 fold increase of primary transcript in all cells, while mRNA was elevated by a similar margin in myoblasts and cardiomyocytes, but not in myotubes (Fig. 3). These cells did not tolerate the long incubation with fatty acids. Following fatty acid exposure, a significant proportion of myotubes appeared damaged upon microscopic inspection. Small increases in H-FABP mRNA could be detected sometimes, but often H-FABP mRNA was reduced or absent, indicative of partial breakdown of messenger RNA.

Subsequently, myoblasts were treated with a series of fatty acids. Saturated and unsaturated long chain-fatty acids all stimulated H-FABP gene expression to similar degrees, both at the level of primary transcript as well as mRNA. After 30 min incubation, a 2–3 fold increase in primary transcript was detected for all fatty acids, with no significant differences due to chain length or degree of desaturation (Fig. 4). H-FABP mRNA also increased in a similar manner after 10 h treatments (Fig. 5).
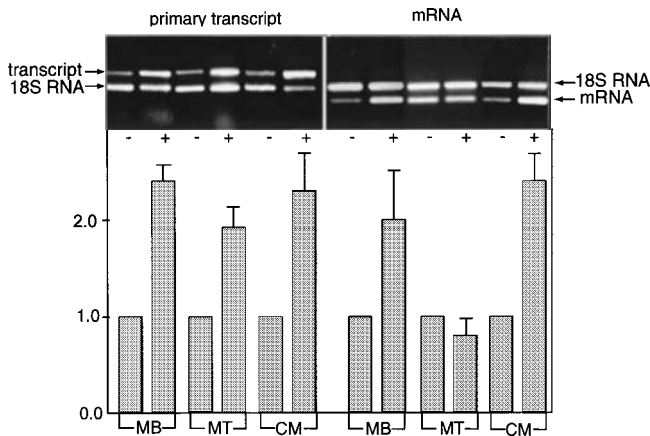
Fig. 3. Induction of H-FABP primary transcript and mRNA levels with linoleic acid. Cells were incubated with culture medium containing 60 μM linoleic acid, complexed to BSA, as described in Materials and methods. Total RNA (500 ng) were used as template for multiplex PCR, with primers specific for 18 S RNA and H-FABP primary transcript (R12/R13, left panel) or mRNA (R1/R4, right panel). MB – myoblasts; MT – myotubes; CM – cardiomyocytes. –, control; +FA, treated with linoleic acid. The 18 S RNA specific primers:competimer ratio was 2:8 for myoblasts and myotubes, 3:7 with primers R12/13, and 4:6 with primers R1/R4. Each value represents the average of 3–6 independent determinations ± S.D.

Discussion

Previous data obtained in whole animals or isolated muscles has long suggested that cardiac FABP expression is up-regulated by fatty acids, but direct experimental proof has only recently been provided in cultured cells. In neonatal cardiomyocytes, van der Lee *et al*. [6] found that H-FABP mRNA increases visibly (2.5-fold) when cultured for 48 h in the presence of 500 μM fatty acid. It was assumed that fatty ac-
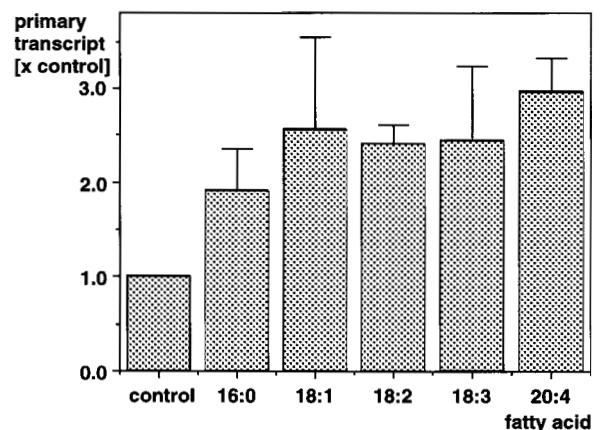
Fig. 4. Induction of H-FABP primary transcript levels in by long chain fatty acids. Cells were treated for 0.5 h with various fatty acids, as described in Materials and methods, and H-FABP primary transcript was amplified by multiplex PCR, as described in Fig. 3. The relative band intensities were determined densitometrically. Each value represents the average of 3–6 independent determinations ± S.D. 16:0 – palmitic acid; 18:1 – oleic acid; 18:2 – linoleic acid; 18:3 – linolenic acid; 20:4 – arachidonic acid.
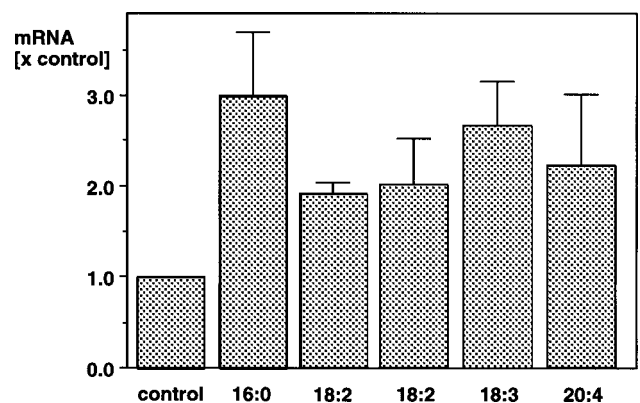
Fig. 5. Induction of H-FABP mRNA levels by long chain fatty acids. Cells were treated for 10 h with various fatty acids, as described in Materials and methods, and H-FABP mRNA was amplified by multiplex PCR, as described in Fig. 3. The relative band intensities were determined densitometrically. Each value represents the average of 3–6 independent determinations ± S.D. 16:0 – palmitic acid; 18:1 – oleic acid; 18:2 – linoleic acid; 18:3 – linolenic acid; 20:4 – arachidonic acid.

ids induce the expression of the H-FABP gene by binding to a transcription factor, possibly a peroxisome proliferator activated receptor (PPAR). However, given the long incubation time and the fact that the levels of various other proteins involved in fatty acid transport and metabolism are also elevated, it is also possible that the increase of H-FABP gene expression depends on fatty acid metabolites or factors affecting its mRNA stability. In this study, we have used a sensitive PCR method to quantify the amount of H-FABP mRNA and of its the primary transcription product which, because of the short half-life of unprocessed RNA, gives an exact measure for the ongoing transcription rate [15]. This has been confirmed for muscle FABP expression in an invertebrate model system [16].

FABP mRNA and its primary transcript are detectable in undifferentiated and differentiated myocytes. When normalized to ribosomal RNA, the increase in H-FABP mRNA levels upon differentiation from myoblasts to myotubes mimics a similar rise in H-FABP expression. Much higher levels of both, primary transcript and mRNA are detectable in isolated adult cardiomyocytes. An exact comparison is not possible, since ribosomal RNA levels vary between the various types of cells. The effect of fatty acid treatment on each cell type, however, can be reliably determined.

Linoleic acid was chosen for the optimization and quantification of fatty acid-mediated induction of gene expression. Linoleic acid is a known activator of other fatty acid inducible genes [17], it is a precursor for other, eicosanoid-derived transcription factor ligands, and is less toxic to cells than saturated fatty acids [18]. In myoblasts, a 2–3 fold induction of H-FABP expression can be seen following incubation times as short as 30 min, while increases in mRNA require 10 or more hours of treatment. A similar increase in H-FABP primary transcript is induced by fatty acid incubation of myotubes, and of isolated adult cardiomyocytes. The increase of the mRNA levels are equally pronounced in myoblasts and cardiomyocytes, but not in myotubes. Generally, myoblasts tolerate a 10 h incubation period well, in contrast to myotubes, which appear to be damaged by longer fatty acid incubations, especially in the absence of horse serum. Previously, it has been reported that myotubes quickly degrade once the horse serum was withdrawn [19], and this may explain the observed lack of induction at the mRNA level. The quantification of the primary transcript provides therefore a more reliable method to study the induction of H-FABP expression.

FABP expression can be induced by various long-chain fatty acids, independent of chain length and degree of desaturation. Similar increases in the H-FABP primary transcript are seen for all fatty acids tested; while these substances also lead to increased mRNA levels after longer incubation periods, the results were more variable. Palmitate often yielded stronger increases than other long chain fatty acids in incubations of up to 10 h. This effect may be due to an accumulation of this fatty acid in the cytoplasm, as long-term incubation with palmitate has been shown to inhibit fatty acid oxidation and induce apoptosis [18].

This study reveals that long-chain fatty acids induce H-FABP gene expression not only in fully differentiated myocytes, but also in myoblasts. Hence, it must be assumed that all factors involved in the fatty acid-mediated gene induction are already present prior to differentiation. Activation of gene expression by fatty acids has also been demonstrated for various other proteins involved in muscle fatty acid transport and metabolism, including fatty acid translocase (CD36), acyl-CoA synthetase, and long-chain acyl-CoA dehydrogenase [6, 12, 20]. However, little is known about the molecular mechanisms by which this stimulation is achieved for muscle specific genes. Better characterized is the control of gene expression by fatty acids in other tissues, especially liver and adipocytes. There is substantial evidence that fatty acids or their metabolites can modulate gene expression at the level of transcription initiation [20] by binding to PPARs that in turn bind to peroxisome proliferator response elements (PPRE), short direct repeat (DR-1) elements upstream of the genes. It appears that PPAR binds to this sequence as a heterodimer with retinoic acid receptor (PPAR/RXR) or other nuclear receptors [21].

While the involvement of these receptors in gene control has been established for a number of proteins involved in lipid-metabolism in adipose and hepatic tissue, functional PPREs have not been demonstrated to control gene expression in muscle tissue, with the exception of a recently identified fatty acid response element (FARE-1) located 775 bp upstream of the muscle carnitine palmitoyltransferase I gene [22]. Potential regulatory sequence with similarity to PPRE consensus sequences have only been found upstream of the H-FABP promoter of rat [23] or mouse H-FABP [24], but not in the other cloned vertebrate (human [25], pig [26] or invertebrate (locust, fruit fly [26]) H-FABP genes. It remains to be seen whether these transcription factors are the fatty acid sensors apparently operational in several other cardiac genes. It may well be possible that other, not yet discovered factors, whether novel forms of PPAR or entirely different proteins, are responsible for the recognition of free fatty acid accumulation in the sarcoplasm. Only detailed reporter gene studies can elucidate this mechanism. Such experiments have not been carried out successfully in differentiated myocytes that are difficult to transfect. Stable or transient transfection, however, is easily achieved in L6 myoblasts. As demonstrated in this study, fatty acids induce H-FABP expression in a similar manner in myoblasts and mature myocytes; thus, myoblasts can serve as a model system to study the molecular mechanisms responsible for the physiologically relevant fatty acid induction of the H-FABP gene in adult cardiomyocytes.

Acknowledgements

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