Inversely related expression of epidermal- and heart-type fatty acid binding proteins during myogenic differentiation of C2C12 myoblasts

Following our report that heart-type (H-) fatty acid binding protein (FABP) was strongly up-regulated in differentiating myotubes, we examined the role of FABP and their effect on oleic acid uptake in the differentiation of mouse C2C12 myoblasts to myotubes in vitro. A second epidermal-type (E-) FABP is already expressed in the myoblast stage and is down-regulated at the onset of myogenic differentiation. This down-regulation is paralleled by that of β-actin, whereas mRNA expression of myogenic transcription factors and of H-FABP increases. These findings were verified at the protein level, which indicated that a basal level of FABPs, independent of the type, is always present during differentiation. Both FABPs were identified by their intracellular binding of oleic acid. This may explain that the change in oleic acid uptake into C2C12 cells at different stages of differentiation was negligible. Yet, the transition from E-FABP expressing myoblasts to H-FABP expressing myotubes was accompanied by a shift in preferential incorporation of oleic acid from triacylglycerols to phospholipids, perhaps necessary for terminal differentiation. The comparison of the expression of H-FABP with that of master myogenic transcription factors demonstrates that this fatty acid binding protein is not involved in the initiation of differentiation. The combination of a particular FABP and the cell in which it is expressed determines the fatty acid uptake.

Keywords: C2C12 cells, myogenic differentiation, fatty acid binding protein, fatty acid uptake, FABP compensation.

1 Introduction

Mammalian H-FABP belongs to a family of highly conserved, mainly cytosolic lipid binding proteins of 14–15 kDa [1–3]. H-FABP is substantially expressed not only in the heart, but also in mammary gland [4], skeletal muscle [5], brain [6], and endothelium of large and small vessels [7]. In rat mammary gland organ cultures this protein inhibited the proliferation of mammary epithelial cells and stimulated their functional differentiation concomitant with milk duct formation [8]. Moreover, a strong up-regulation of H-FABP at transcriptional [9] and translational [4, 10] levels in the course of differentiation from virgin to lactating mammary gland suggested that H-FABP is a differentiation factor [11]. Against this background we tested H-FABP as a differentiation factor in muscle development employing the mouse C2C12 cell culture model, where proliferating mononucleated myoblasts can be stimulated to differentiate into the post-mitotic polynucleated myotubes by withdrawal of growth factors from the culture medium. We demonstrated that H-FABP was present in C2C12 myoblasts in minute amounts only, but its expression increased with differentiation resulting in elevated protein levels in myotubes [12].

With respect to lipid metabolism, FABPs are involved in fatty acid uptake and transport, intracellular utilization, and targeting of fatty acids, as well as in modulation of enzyme activities (reviewed in [2]). In this report we tested the hypothesis that intracellular H-FABP concentration affects the uptake of fatty acids in a myogenic cell line as it does in cardiac myocytes [13]. We expected the induction of H-FABP expression to be paralleled by enhanced fatty acid uptake in the course of myogenic differentiation of C2C12 cells. However, our data were at variance with this hypothesis. Thus, we reinvestigated the time-dependent expression of H-FABP during the conversion of C2C12 myoblasts into myotubes by comparing the transcription of H-FABP with that of the myogenic transcription factors. We then checked for the presence of other FABPs as potential intracellular binding proteins for fatty acids in order to explain the constant fatty acid uptake during myogenic differentiation.

2 Materials and methods

2.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM), phosphate buffered saline (PBS, without Ca²⁺, Mg²⁺), serum
and glutamine were supplied by Biochrom (Berlin, Germany). Insulin, the substrates for Western blot detection, T7 and SP6 RNA-polymerases, DIG-UTP and restriction enzymes were from Roche Diagnostics (Basel, Switzerland). Oligonucleotides for PCR were purchased from MWG Biotech (Ebersberg, Germany), nitrocellulose membrane from Schleicher & Schuell (Dassel, Germany). Sephacryl S-100 HR was purchased from Pharmacia (Freiburg, Germany) and [1-14 C]oleic acid (58 Ci/mol) from Amersham (Braunschweig, Germany). Silica gel 60 F254 plates for high performance thin-layer chromatography (HPTLC) were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA, fraction V, essentially fatty acid free), anti-rabbit IgG alkaline phosphatase conjugate and all other analytical grade chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2 Cell culture

Mouse C2C12 myoblasts (ATCC, CRL-1772), obtained from the American Type Tissue Culture Collection (Rockville, MD, USA), were maintained in growth medium (DMEM with 4.5 g/l glucose, 15% fetal calf serum, 4 mM glutamine). For induction of differentiation, cells were seeded with 5000 cells/cm² one day before the growth medium was exchanged by the differentiation medium (DMEM with 4.5 g/l glucose, 1% horse serum, 4 mM glutamine, 0.3 µM insulin). The withdrawal of serum is accompanied by a reduction of fatty acid concentration in the medium and could have had an effect on gene regulation. This we can exclude as both, E- and H-FABP do not contain functional peroxisomal proliferator responsive elements to which peroxisome proliferator activated receptors bind upon interaction with fatty acids (Bleck, Schachtrup and Spener, unpublished results). During the course of cell differentiation 3 stages were defined: (i) The mononucleated muscle precursor cells (myoblasts), (ii) from the onset of myoblast fusion to the first polynucleated myotubes at days 4 and 5 (early myotubes), and (iii) the more mature myotubes at day 7 and later (late myotubes).

2.3 RNA isolation and Northern blot analysis

Total RNA was extracted from C2C12 cells using the method of Chomczynski et al. [14] and separated on a 1% agarose gel containing 5% (v/v) formaldehyde. The RNA was then transferred onto a positively charged nylon membrane (Roche Diagnostics) [15]. Hybridization with DIG-labeled DNA-probes and detection of RNA with chemiluminescence substrate CDP-Star was carried out according to the manufacturer’s instructions. Signals on the film, each in the linear portion of the gray scale, were quantified by laser-scanning densitometry (UltraScan XL, Pharmacia) and normalized to the signal of the constitutively expressed 7S-RNA [16].

2.4 Antisense RNA probe preparation

Antisense RNA probes were generated by a combination of RT-PCR and in vitro transcription. Total RNA from C2C12 myoblasts and myotubes was reversely transcribed with the first-strand cDNA synthesis kit (Pharmacia). The cDNA-pool obtained served as template in a PCR introducing a T7 or SP6 RNA-polymerase site in antisense orientation with respect to the resulting PCR-fragments. The primer sequences for each probe, the length of resulting PCR-fragments and references to corresponding cDNAs are presented in Tab. 1. For murine H-FABP and E-FABP, antisense RNA probes were generated from the more specific 3’-untranslated regions. Therefore pUC18-mH-FABP [12] and a 11 kbp subclone of the Fabpe gene [17], respectively, were used as templates in the PCR. In vitro transcription reactions were carried out with 250 ng of the corresponding amplicon with T7 or SP6 RNA-polymerase, respectively, and with DIG-UTP as label. After ethanol precipitation the DIG-labeled antisense RNA probes were taken up in formamide, quantified spectrophotometrically and stored at –70 °C until use.

Tab. 1. Generation of antisense RNA probes.

<table>
<thead>
<tr>
<th>antisense RNA probe</th>
<th>5’-primer</th>
<th>3’-hybrid primer a</th>
<th>PCR-fragment length [bp]</th>
<th>mRNA size [nt]</th>
<th>cDNA reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyoD1</td>
<td>AGTGAATGAGGCGCTTCGAGA</td>
<td>T7-ATGCGGCTCCACTATGCTGGA</td>
<td>422</td>
<td>2200</td>
<td>[56]</td>
</tr>
<tr>
<td>myogenin</td>
<td>AGTGGCAGGAAACAGGCTTTT</td>
<td>T7-CTGGCGCAGGATCTCCACCTT</td>
<td>420</td>
<td>1600</td>
<td>[57]</td>
</tr>
<tr>
<td>actin</td>
<td>ACGTGCTTGCAGATGATGATG</td>
<td>T7-CAACAGGAAATAGCCACGC</td>
<td>239</td>
<td>α-1700 β-2000</td>
<td>[35]</td>
</tr>
<tr>
<td>HAD</td>
<td>CCTGTTCTGCTCTAGAAC</td>
<td>T7-CTCTTGTGATATCGTCTCCTG</td>
<td>290</td>
<td>2900</td>
<td>[58]</td>
</tr>
<tr>
<td>7S-RNA</td>
<td>GCCGTGATTGCGCTGATCTA</td>
<td>SP6-CTCGGCTGCTGATCCACCTC</td>
<td>196</td>
<td>280</td>
<td>[16]</td>
</tr>
<tr>
<td>H-FABP</td>
<td>ATGCGGCGAGCGCTTCG</td>
<td>T7-ACCATGAGGACAGCATG</td>
<td>500</td>
<td>830</td>
<td>[12]</td>
</tr>
<tr>
<td>E-FABP</td>
<td>GAGTGTGTGATGAAAAAG</td>
<td>T7-TACTAACCAGAAAGAATG</td>
<td>270</td>
<td>860</td>
<td>[33]</td>
</tr>
</tbody>
</table>

aRNA polymerase promoter sequences: T7, TAATACGACTCAGACTAGGG; SP6, GATTAGGTGACACTATAG.
2.5 Cloning of murine E-FABP from C2C12 myoblasts and heterologous expression in E. coli

Total RNA from C2C12 myoblasts was reversely transcribed with the first-strand cDNA synthesis kit (Pharmacia). The cDNA-pool obtained served as template in a PCR introducing 5'-Nde I and 3'-Bam HI restriction sites. After cloning into pCRScript (Stratagene) the insert was subjected to double-stranded sequencing to assure the identity of the cDNA. The E-FABP cDNA was cloned into pET-20b vector for expression in E. coli-BL21 (DE3) pLysS. The protein was expressed and purified to homogeneity (>95% purity, assessed by SDS polyacrylamide gel-electrophoresis) by a combination of ion-exchange and size-exclusion chromatography essentially as described for the purification of recombinant human E-FABP [18], with a yield of 33 mg/l broth.

2.6 Western blot analysis and protein quantification

C2C12 supernatant (14,000 × g) was obtained as described earlier [12]. Total protein concentrations were determined according to Smith et al. [19]. Cell supernatant was resolved by SDS polyacrylamide gel-electrophoresis (15% C, 2.7% T) together with different amounts of recombinant H-FABP and E-FABP (5–50 ng each with 1 mg/ml BSA as carrier protein on the same gel. Western blotting was done essentially as described earlier [20] using polyclonal rabbit antibodies against rat E-FABP [20] and murine H-FABP [21]. Protein bands from sample lanes were quantified by comparison with the FABP standards loaded in adjacent lanes on the same blot. Therefore, the nitro-cellulose sheet was immersed with light mineral oil [22] and the resulting signals were analyzed by laser-scanning densitometry as described in section 2.3.

2.7 Fatty acid uptake

For uptake studies, C2C12 cells were cultured up to the differentiation stages in 25-cm² flasks as described in section 2.2. Cell monolayers were washed twice with DMEM (37°C) and incubated with 100 µM oleic acid, bound to 33 µM BSA, in DMEM containing 12.5 mg/l gentamycin. This fatty acid/BSA ratio of 3 resulted in a physiological free fatty acid concentration of 20–30 nM [23], this concentration showed highest overall uptake rates within the linear range. Prior to incubation [1-14C]oleic acid was diluted with cold oleic acid to give a final specific activity of 1.5 Ci/mol. The uptake of oleic acid by C2C12 cells was linear between 5 and 90 min, i.e. the uptake rate was constant within this time range (data not shown). Actual incubation times chosen for measuring oleic acid uptake rate were at 30 and 60 min. For termination the incubation medium was aspirated and cells were washed 3 times with ice-cold PBS containing 0.2 mM phloretin as a non-selective inhibitor of membrane transport. After the third washing step no residual radioactive oleic acid was detected in the wash solution. Cells were scraped off and lysed in distilled water by passing them 10 times through a syringe needle. Each lysate was subjected to scintillation counting and protein determination [19].

2.8 Lipid extraction and separation by HPTLC

Total lipids were extracted from C2C12 lysates according to Bligh and Dyer [24], solvents removed under nitrogen and the residue taken up in chloroform. For separation of lipids, HPTLC-plates were first developed in chloroform-methanol-acetic acid-formic acid-water 35:15:6:2:1 (v/v) up to the half-length of the plate and then in hexane-diisopropyl ether-acetic acid 65:35:2 (v/v) over the whole plate [25]. For investigation of lipid composition the plates were sprayed after evaporation of the solvents with 3% cupric acetate / 8% phosphoric acid, charred at 120 °C for 15 min [26] and resulting signals were analyzed by laser-scanning densitometry as described above (section 2.3). For examination of oleic acid incorporation into lipid classes, HPTLC plates were charred at 120 °C for 15 min after evaporation of the solvents and incorporation of radioactive label was analyzed by exposing X-ray films (Hyperfilm ECL, Amersham) to the plates at –70 °C for 7 to 9 d and subsequent evaluation of resulting signals by laser-scanning densitometry as described above.

2.9 Co-elution of FABPs and [1-14C]oleic acid

To investigate whether in C2C12 cells oleic acid is intracellularly bound to FABPs we modified the co-elution method of Samanta et al. [27]. First we examined whether a Sephacryl S-100 HR column (25 x 1.7 cm, Pharmacia) at a flow rate of 1 ml min⁻¹ can separate soluble proteins in the low molecular mass range. Only ice-cold buffers were applied to the column to ‘freeze’ the binding of the fatty acids at their equilibrium, while the separation was carried out at room temperature. Thus, the column was equilibrated with 5 volumes of PBS and 1 mg of each, recombinant murine H-FABP (15 kDa) and chymotrypsin (25 kDa) or murine E-FABP and chymotrypsin in 200 µl PBS were applied. One ml fractions were collected and aliquots were subjected to protein determination [19] and FABP identification by Western blotting as described in section 2.6. A baseline separation of respective FABP and chymotrypsin was attained with E-FABP eluting at 16 ± 2 min, H-FABP at 19 ± 2 min, chymotrypsin at
54 ± 3 min and unbound oleic acid at 7 ± 2 min (data not shown).

Then 14,000 x g supernatants were prepared from C2C12 myoblasts and late myotubes (day 7) as described earlier [12]. Supernatant protein (200 µg) in 100 µl PBS was incubated at 37 °C for 30 min with 1 nmol [1-14C]oleic acid dissolved in 100 µl PBS, then cooled on ice for 15 min and applied to the column equilibrated with ice-cold PBS, and separated as described above. Aliquots of each 1 ml fraction collected were subjected to scintillation counting and normalized to the peak of highest radioactivity, which was set to 1.

2.10 Statistical analysis

First the null-hypothesis was tested, if the means of a data group were equal (at a significance level of p < 0.05), by one way analysis of variance (ANOVA). Groups rejecting the null-hypothesis were subjected to post-hoc Newman-Keuls analysis (p < 0.05).

3 Results

3.1 Expression pattern of mRNAs encoding marker proteins of myogenic differentiation

To classify the differentiation dependent expression of H-FABP in our muscle cell model in comparison to that of genes relevant for muscle differentiation and lipid metabolism, Northern blot analyses were carried out (Fig. 1). No mRNA for myogenic transcription factor myogenin was detected in subconfluent myoblasts, whereas at early onset of differentiation a strong increase of myogenin mRNA was observed. In contrast, MyoD1 mRNA, coding for another myogenic transcription factor, showed a relatively constant expression during differentiation with a clear signal in the muscle precursor cells already, characteristic for its role in the myogenic cell determination (Fig. 1A). Actin mRNAs revealed a switch from non-muscle β-actin to contractile α-actin during myogenic differentiation, indicating a phenotypic change (Fig. 1B). The transcript for β-hydroxyacyl-CoA dehydrogenase (HAD) was seen in myoblasts, but its concentration increased gradually during myogenic differentiation (Fig. 1C). In comparison to the differentiation dependent increase of myogenin and α-actin mRNAs induction of H-FABP mRNA was rather late (Fig. 1D). These data clearly revealed that H-FABP is not involved in triggering muscle cell differentiation.

3.2 Fatty acid uptake into differentiating C2C12 muscle cells and their incorporation into lipids

The uptake of oleic acid into the cultured C2C12 cells, measured after 30 and 60 min, was examined to test if there were factors that would influence oleic acid utilization. Cell monolayers at different stages of differentiation were incubated with radioactively labeled oleic acid complexed to BSA as described under Materials and Methods. At the time points measured oleic acid uptake was linear. Uptake rates were determined after 30 min (n = 3) and 60 min (n = 5). As there was no difference the respective data for each differentiation stage results were lumped together and are expressed as means ±SD. Uptake rate of oleic acid into C2C12 myoblasts was 2.35 ± 0.58 nmol (min mg protein)-1, into early (day 4) myotubes 3.44 ± 0.87 nmol (min mg protein)-1 and into late (day 7) myotubes 2.77 ± 0.74 nmol (min mg protein)-1. There was no significant change in [1-14C]oleic acid uptake in response to differentiation.
Next we investigated the lipid composition of the cells in the 3 differentiation stages (Tab. 2). There were remarkable changes as a result of differentiation from myoblasts to myotubes, i.e. a significant 2- to 3-fold increase in the proportion of phospholipid classes (phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine) and a 2.5-fold decrease of the proportion of triacylglycerols and cholesterol esters. The 1.4-fold decrease observed for diacylglycerols was not significant. There were only minor differences in lipid composition of early and late myotubes.

The uptake of [1-14 C]oleic acid in cells in the 3 differentiation stages was examined (Tab. 3). The 60 min time point was chosen because only a very small portion of the fatty acid taken up could still be recovered from the cells in unmetabolized form [28, 29]. Three quarters of the 14C-label incorporated was found in phosphatidylcholine, triacylglycerols and fatty acids. In line with the observed changes in lipid composition during myogenic differentiation of C2C12 cells the synthesis of phospholipid classes was between 1.4- and 5.9-fold higher in late myotubes than in myoblasts, whereas myoblasts showed a significant decrease observed for diacylglycerols was not significant. There were only minor differences in lipid composition of early and late myotubes.

Tab. 2. Changes of cellular lipid composition during myogenic differentiation of C2C12 cells. Cells were cultured in monolayers to the corresponding differentiation stages, lipids were extracted and separated into lipid classes by HPTLC as described in Materials and methods. After spraying and charring lipid classes were analysed by densitometric scanning of HPTLC plates. All data are means ± SD (n = 5).

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Myoblasts [% of total lipids]</th>
<th>Early myotubes [% of total lipids]</th>
<th>Late myotubes [% of total lipids]</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysophosphatidylcholine</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.5</td>
<td>1.6 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>3.7 ± 0.5</td>
<td>2.7 ± 1.5</td>
<td>3.0 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>5.8 ± 6.0</td>
<td>16.7 ± 4.5</td>
<td>18.8 ± 2.1</td>
<td>a</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.7 ± 0.4</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>a,b</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>0.9 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>a,b</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>3.6 ± 2.4</td>
<td>8.9 ± 2.7</td>
<td>8.8 ± 4.7</td>
<td>a</td>
</tr>
<tr>
<td>Cerebrosides</td>
<td>2.3 ± 2.1</td>
<td>1.8 ± 2.0</td>
<td>2.1 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>1,2-Diacylglycerols</td>
<td>3.7 ± 0.8</td>
<td>2.2 ± 1.5</td>
<td>2.6 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>1,3-Diacylglycerols</td>
<td>9.3 ± 0.1</td>
<td>4.4 ± 2.1</td>
<td>6.4 ± 1.5</td>
<td>a</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>23.4 ± 12.2</td>
<td>29.8 ± 3.3</td>
<td>30.8 ± 1.8</td>
<td>ns</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>4.7 ± 3.5</td>
<td>4.4 ± 1.6</td>
<td>5.4 ± 1.4</td>
<td>ns</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>4.7 ± 0.4</td>
<td>2.9 ± 0.9</td>
<td>1.9 ± 0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>35.7 ± 1.4</td>
<td>22.9 ± 10.5</td>
<td>15.7 ± 7.0</td>
<td>a</td>
</tr>
</tbody>
</table>

ns – not significant (ANOVA), a significant difference compared to myoblasts (p < 0.05), b significant difference compared to early myotubes (p < 0.05).

Tab. 3. Change in de novo lipid synthesis during myogenic differentiation of C2C12 cells. Cells were cultured in monolayers to the corresponding differentiation stage and incubated with 100 µM [1-14 C]oleic acid bound to 33 µM BSA for 60 min. Total lipid were extracted and separated into lipid classes by HPTLC as described in Materials and methods. Incorporation of radioactive label into lipid classes was analyzed by densitometric scanning of autoradiograms of respective HPTLC plates. All data are means ± SD (n = 5).

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Myoblasts [% of total radioactivity incorporated]</th>
<th>Early myotubes [% of total radioactivity incorporated]</th>
<th>Late myotubes [% of total radioactivity incorporated]</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>22.9 ± 7.0</td>
<td>31.6 ± 3.3a</td>
<td>32.1 ± 4.8a</td>
<td>a</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.8 ± 1.7</td>
<td>5.3 ± 0.9a</td>
<td>5.0 ± 1.1a</td>
<td>a</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>–</td>
<td>0.7 ± 0.4a</td>
<td>2.0 ± 0.4a,b</td>
<td>a</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>2.5 ± 1.4</td>
<td>5.7 ± 1.3a</td>
<td>6.5 ± 2.9a</td>
<td>a</td>
</tr>
<tr>
<td>Cerebrosides</td>
<td>0.6 ± 1.2</td>
<td>1.2 ± 0.5</td>
<td>1.8 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>1,2-Diacylglycerols</td>
<td>5.1 ± 2.7</td>
<td>3.6 ± 1.8</td>
<td>5.8 ± 3.3</td>
<td>ns</td>
</tr>
<tr>
<td>1,3-Diacylglycerols</td>
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<td>10.3 ± 2.5</td>
<td>6.9 ± 5.3</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.0 ± 4.4</td>
<td>2.1 ± 1.1</td>
<td>1.2 ± 0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>20.2 ± 6.5</td>
<td>22.3 ± 2.2</td>
<td>23.1 ± 6.8</td>
<td>ns</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>31.9 ± 6.4</td>
<td>16.0 ± 6.1a</td>
<td>14.5 ± 8.1a</td>
<td>a</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>–</td>
<td>0.8 ± 1.1</td>
<td>0.9 ± 1.3</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns – not significant (ANOVA), a significant difference compared to myoblasts (p < 0.05), b significant difference compared to early myotubes (p < 0.05).
cant higher synthesis (2.2-fold) of triacylglycerols. Interestingly, small amounts of the radioactive label were also found in cholesterol, indicating that the oleic acid taken up into the cells was also fully metabolized to C2 units and reused for de novo cholesterol synthesis during the 60 min. The observed decrease of incorporation of label into cholesterol was not statistically significant, however. Taken together, the incorporation of oleic acid in late myotubes parallels the increase of phospholipids and decrease of triacylglycerols, indicating a switch in the de novo synthesis of lipids during the course of myogenic differentiation.

3.3 Differentiation dependent expression of E-FABP in C2C12 cells

This unexpected data on fatty acid uptake prompted us to screen C2C12 cells for further FABP-types present, in particular in myoblasts. Intracellular presence of another FABP-type could compensate for the lack of H-FABP. FABP-types such as adipocyte-(A-), brain-(B-) and intestinal-(I-) FABP have restricted expression patterns and have not been found in muscle cells [1, 30]. Thus, they were not considered candidates for expression in C2C12 cells. We considered liver-(L-) FABP, which has been found to be co-localized with H-FABP in other tissues (reviewed in [31]), but it was not detected in C2C12 cells by means of a highly sensitive ELISA [32] (data not shown).

Since E-FABP was first detected in and cloned from the epidermis [33, 34] and found later in a variety of other tissues and cell types (reviewed in [30]) we focused our attention on this protein. Consequently, for application in quantitative protein analysis we cloned murine E-FABP from C2C12 myoblasts and the sequence of cDNA obtained was identical to that of murine E-FABP isolated from mouse epidermis and originally named ‘mal1’ [33]. Heterologous expression of the protein in E. coli and purification afforded the valuable standard protein for Western blotting. The affinity-purified polyclonal rabbit antibody against rat E-FABP (92% identical to murine E-FABP) was able to detect recombinant murine E-FABP down to 5 ng. As shown in Fig. 2, E-FABP content was highest in supernatants of C2C12 myoblasts (0.49 ± 0.11 µg (mg protein)−1) and decreased to 0.09 ± 0.04 µg (mg protein)−1 during the course of differentiation. H-FABP, detectable in minute amounts, if at all in myoblasts (0.003 ± 0.0004 µg (mg protein)−1), peaked in myotubes at day 9 (0.45 ± 0.11 µg (mg protein)−1).

Turning to the transcriptional level we generated a specific antisense E-FABP mRNA probe using the 3’-untranslated region. Using this probe in Northern blot analysis of total RNA from differentiating C2C12 cells revealed a strong expression of E-FABP mRNA (860 ± 50 nt) in myoblasts which, upon differentiation to myotubes, declined to a weak signal at day 7. After scanning and normalizing the signals of 3 independent Northern blots to constitutively expressed 7S-RNA, a dramatic decrease of the E-FABP mRNA content during the course of myogenic cell differentiation was seen (Fig. 1D). Direct comparison to H-FABP transcription revealed an inversely related expression pattern for the two FABP-types in differentiating C2C12 muscle cells (Fig. 1D).

3.4 Intracellular binding of oleic acid by FABPs

The unchanged rates of oleic acid uptake rates into myoblasts, early and late myotubes could therefore be due to the presence of at least one of the two FABP-types in the respective cells. To test oleic acid binding to these proteins we incubated the 14,000 × g supernatants of E-FABP expressing myoblasts and H-FABP expressing late myotubes with [1-14C]oleic acid, and checked, upon separation of the supernatant proteins by FPLC, the co-elution of proteins with [1-14C]oleic acid and, checked, upon separation of the supernatant proteins by FPLC, the co-elution of proteins with [1-14C]oleic acid. In both cases only one radioactive peak was observed at 18 ± 2 min and 24 ± 2 min for myoblasts and myotubes, respectively (Fig. 3). The retention times can be correlated to the retention times of recombinant E- and H-FABP eluting at 16 ± 2 min and 19 ± 2 min, respectively, used for calibra-
tion of the column. To further verify oleic acid binding to FABPs we added 2 µg recombinant FABP (E-FABP to myoblasts, H-FABP to myotubes) to the respective supernatant (200 µg protein). As expected this resulted in one peak only in either case (data not shown). Due to different matrices, retention times for FABPs in respective supernatants were slightly higher than those for E- and H-FABP applied in PBS for calibration.

4 Discussion

Using the C2C12 cell line as in vitro model for myogenesis we detected and identified recently H-FABP [12] and now E-FABP at the protein and mRNA level. With the aid of a specific antisense E-FABP RNA probe we detected the smaller (850 nt) of the two known E-FABP transcripts. This is in line with findings that the 850 nt mRNA predominate, also in skeletal muscle [33].

Our detailed analysis comprised transcription patterns of myogenic transcription factors MyoD1 and myogenin. The former is known to determine the myogenic lineage, the latter is strongly induced at the onset of differentiation. These patterns and the switch in expression of the actins is in accordance with data reported for the C2C12 cell line as an in vitro model for myogenesis [35, 36]. The expression of E-FABP in C2C12 cells is down-regulated at the transcriptional level at the onset of myogenic differentiation, similar to non-muscle β-actin. This was reflected by the 50% decrease of E-FABP mRNA already at day 3 and by its disappearance at day 6 of differentiation. Either E-FABP mRNA is expressed in decreasing concentrations in myotubes, or the decrease is due to E-FABP mRNA being exclusively expressed in myoblasts. In the latter case E-FABP mRNA would then be detected in the myotube culture due to myoblast background, which persists even after 10 d in differentiation medium. In any case this is the first observation of the differentiation-dependent down-regulation of E-FABP. Previously only a strong up-regulation in epidermis during tumor formation was reported [33].

In comparison to the early players in myogenic differentiation mentioned above, H-FABP mRNA started to increase only after day 2, when the first morphological changes in the cells were evident. Thus H-FABP may be considered a factor in terminal muscle cell differentiation. Indeed, remarkable changes in lipid composition and specific incorporation of radioactively labeled oleic acid into different lipid classes were seen in myoblasts compared to myotubes. We also observed an increase in the proportion of phospholipids and a decrease in the proportion of triacylglycerols and cholesterol esters during differentiation (Tab. 2). The incorporation of radioactivity from [1-14C]oleic acid also showed a higher incorporation into phospholipids in late myotubes than in myoblasts, whereas myoblasts synthesize a significant higher amount of triacylglycerols (Tab. 3). It is possible that E-FABP preferentially directs oleic acid towards incorporation into triacylglycerols, whereas H-FABP channels this fatty acid towards preferential incorporation into phospholipids. This could mean that the switch in FABP expression affects fatty acid utilization by C2C12 cells during differentiation. This is still highly speculative, but a precedent has been reported in the literature. In transfected mouse L-cell fibroblasts I-FABP directs oleic acid to triacylglycerols and L-FABP to phospholipids [28, 37–39].

The elevated phospholipid content and synthesis in C2C12 myotubes is most likely related to the known increase in membrane synthesis (e.g. sarcoplasmic reticulum) after myoblast fusion. In contrast fatty acids in myoblasts may be temporarily stored as esters in triacylglycerols before being used for synthesis and re-modelling of membrane phospholipids. Thus H-FABP may actually play a role in myogenic differentiation by making substrates available for increased membrane synthesis necessary for terminal differentiation.

Recently, a similar induction of an extracellular lipocalin (Ex-FABP also known as CH21) with fatty acid binding activity in differentiating chicken muscle cells has been reported [40]. It is not known up to date whether a homologous protein is present in mammals as well.
Interestingly, the transcription pattern of HAD, a member of the mitochondrial β-oxidation multienzyme complex, showed a modest, but significant (at a level of p < 0.05) increase of the messenger signal as H-FABP became up-regulated. In line with these data are reports on the correlation between fatty acid oxidation rate and H-FABP content in rat heart and quadriceps muscle [41, 42]. The spontaneously contracting C2C12 cells were derived from mouse soleus, a muscle known to consist of mainly slow-twitch oxidative muscle fibers. Their energy requirements are primarily met by fatty acids oxidation [43]. However, it remains to be elucidated whether myotubes meet their energy demand predominantly via β-oxidation.

Examples of the co-existence of two or more FABP types in one cell-type are known, e.g. glial cells, fibroblasts of lung origin, and adipocytes [20, 21, 44]. In the 3T3-L1 adipogenic cell line A- and E-FABP are detected already in the preadipocyte stage and both are up-regulated during differentiation into adipocytes [44]. Interestingly, in adipocytes isolated from wild-type mice low amounts of E-FABP and high amounts of A-FABP are expressed, but a 10-fold increase in E-FABP expression was observed in case of adipocytes from A-FABP knock out mice [45]. In this knock out model E-FABP may compensate for the lack of A-FABP. It appears that the inversely related expression of E- and H-FABP in differentiating C2C12 cells is an excellent cellular model for the compensatory potential attained by FABPs in fatty acid transport.

Fatty acid uptake is a complex process that depends on membrane translocation of the fatty acid, either by passive diffusion and/or by active transport, mediated by membrane-associated fatty acid translocators [5, 46], and on their subsequent utilization which depends on the physiological status of the cells. Initial fatty acid uptake is considered to be driven mainly by membrane-associated fatty acid translocators [5, 47]. In our study we measured uptake of fatty acids over 60 min as it is linked to their intracellular utilization. The role of FABPs in this uptake process is equivocally described in the literature. After transfection of murine L-cell fibroblasts with rat L-FABP cDNA the expression of L-FABP increased the initial uptake rate for oleic acid up to 80% [39]. Analysis of human HepG2 hepatocarcinoma cells with manipulated L-FABP content revealed a direct correlation between L-FABP concentration and fatty acid uptake [32]. It was also demonstrated that the fatty acid uptake was elevated in CHO-cells transfected with wild-type A-FABP in contrast to non-binding A-FABP mutants [48]. Other findings, however, indicated that the enhancing effect of FABP(s) on fatty acid uptake might not be a general principle. First, rat hBRIE 380 cells, derived from intestinal epithelium [49], mouse L-cell fibroblasts [28] and enterocyte-like human Caco-2 cells [50], which may or may not express any endogenous FABP, revealed no increase in fatty acid uptake as a result of transfection with I-FABP. Second, expression of rat L-FABP mRNA in Xenopus laevis oocytes did not increase the fatty acid uptake rate of these cells [51]. Our experiments with C2C12 cells revealed no significant difference between myoblasts, early myotubes or late myotubes with regard to fatty acid uptake rates. A role of H-FABP in fatty acid uptake has become apparent. A significantly reduced cardiac uptake of a non-metabolizable fatty acid analog is observed in H-FABP deficient mice [52]. Similarly isolated cardiac myocytes from wild-type and H-FABP deficient mice differ in palmitate uptake, reduced in H-FABP deficient myocytes [13]. This does not contradict our results in differentiating C2C12 cells. An increased, compensatory FABP-expression was not observed in cardiomyocytes of H-FABP deficient mice [8, 13]. We conclude that E- and H-FABP together ensure that oleic acid uptake in myoblasts and myotubes remains constant. In the light of this result one may take a new look at data reported for the differentiating rat muscle cell line L6, in which H-FABP content [53] and the fatty acid oxidation rate [54] increased, whilst the uptake of fatty acids remained unchanged [53]. A compensatory increase in E-FABP levels may account for this observation. The significant 67% increase in oleic and palmitic acid uptake rates in MCF7 human breast cancer cells transfected with bovine H-FABP may have been caused by a compensatory effect of E-FABP as well. The presence of E-FABP was demonstrated [55]. In view of this discussion we can conclude that the effect of FABP(s) on cellular fatty acid uptake seems to be a specific function of the combination of a particular cell- and FABP-type. In addition the structure of fatty acids is also important, since FABP binding affinities vary for different fatty acids [2].

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