Statins and fibrates can cause myopathy. To further understand the causes of the damage we performed a proteome analysis in fast-twitch skeletal muscle of rats chronically treated with different hypolipidemic drugs. The proteomic maps were obtained from extensor digitorum longus (EDL) muscles of rats treated for 2-months with 10 mg/kg atorvastatin, 20 mg/kg fluvastatin, 60 mg/kg fenofibrate and control rats. The proteins differentially expressed were identified by mass spectrometry and further analyzed by immunoblot analysis. We found a significant modification in 40 out of 417 total spots analyzed in atorvastatin treated rats, 15 out of 436 total spots in fluvastatin treated rats and 21 out of 439 total spots in fenofibrate treated rats in comparison to controls. All treatments induced a general tendency to a down-regulation of protein expression; in particular, atorvastatin affected the protein pattern more extensively with respect to the other treatments. Energy production systems, both oxidative and glycolytic enzymes and creatine kinase, were down-regulated following atorvastatin administration, whereas fenofibrate determined mostly alterations in glycolytic enzymes and creatine kinase, oxidative enzymes being relatively spared. Additionally, all treatments resulted in some modifications of proteins involved in cellular defenses against oxidative stress, such as heat shock proteins, and of myofibrillar proteins. These results were confirmed by immunoblot analysis. In conclusions, the proteomic analysis showed that either statin or fibrate administration can modify the expression of proteins essential for skeletal muscle function suggesting potential mechanisms for statin myopathy.
small GTP-binding proteins involved in myocytes preservation [14]. Different studies have shown that statin and fibrate can modify gene and protein expression in skeletal muscle [15]. For instance, an up-regulation of ryanodine receptor, suggestive of intracellular calcium increase, was found in muscle biopsies of statin treated patients showing evident structural damage [16]. Our previous studies have demonstrated that statin and fibrate affect skeletal muscle function also by modifying calcium homeostasis and resting chloride conductance (gCl). Indeed, lipophilic statin increased intracellular calcium via mitochondria and sarcoplasmic reticulum release [17], thereby affecting contractile function. In turn these drugs reduced resting gCl [18,19], a parameter sustained by the CIC-1 chloride channel and modulated by calcium-dependent PKC [20–25]. This parameter is normally high in fast-twitch muscles and is important to guarantee muscle membrane potential and excitability [20–22].

2. Methods

2.1. Animal care and treatments

The animal study protocol was conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conforms to the European Community Directive published in 1986 (86/609/ EEC). Adult male Wistar rats (Charles River Laboratories, Calco, Italy), weighing 300–350 g, were housed individually in appropriate metabolic cages in an environmentally controlled room and received commercial rodent chow (30 g/day) (Charles River, 4RF21) and water ad libitum. Rats were randomly assigned to 4 experimental groups of 8 animals each: (1) fluvastatin 20 mg/kg/day treated animals (FLUVA), (2) atorvastatin 10 mg/kg/day treated animals (ATO), (3) fenofibrate 60 mg/kg/day treated animals (FENO), (4) control animals (CTRL) treated with the vehicle (0.5% carboxymethylcellulose in aqueous solution) used to dissolve the drugs. Fluvastatin (Lescol, Novartis), atorvastatin (Torvast, Pfizer), and fenofibrate (Lipsin, Cabrera) were administrated orally by using an esophageal cannula, once a day for two months [20]. During the treatment the body weight and vital parameters (health conditions, water and food consumption) were normal in all treated rats. Skeletal muscle performance was evaluated daily by testing in each rat the righting reflex, i.e. the ability of the rat to straighten itself on four legs when turned on the back. The observation of the righting reflex can help to detect severe myotonic-like signs or alteration of muscle function. As previously observed, the righting reflex was normal during the entire treatment period in all the animals. No mortality was observed. At the end of the 2-months treatment rats were sacrificed by cervical dislocation and the extensor digitorum longus (EDL) muscle was carefully dissected from each rat and immediately frozen in liquid nitrogen, then stored at –80 °C until proteomic analysis. The contralateral EDL muscle of all fluvastatin, atorvastatin and fenofibrate treated rats was immediately placed in a appropriate muscle bath chamber to measure the resting chloride conductance (gCl) by the 2-intracellular microelectrode technique [20]. Histological analysis was also performed on four tibialis anterior muscles dissected from randomly selected treated rats of each experimental group as previously described [24].

2.2. Proteome analysis (2-DE)

2.2.1. Sample preparation

The methods of proteome analysis are mostly the same as those previously used [29]. Muscle samples previously stored at –80 °C, were pulverized in a steel mortar with liquid nitrogen to obtain a powder that was immediately resuspended in a lysis buffer [8 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 40 mM Tris base (Healthcare, Germany) and a cocktail of protease inhibitors (Sigma–Aldrich, Italy)]. The samples were vortexed, frozen with liquid nitrogen and thawed at room temperature four times; then the samples were incubated with Dnase and Rnase for 45 min at 4 °C to separate proteins from nucleic acids and finally spun at 35,000 × g for 30 min. Protein concentration in the dissolved samples was determined with a protein assay kit (2D quant Kit, Healthcare). In order to perform proteome analysis, a sample mix was obtained for each experimental group (CTRL, FLUVA, ATO, FENO). Each sample mix contained an equal protein quantity taken from each muscle sample of CTRL, FLUVA, ATO, FENO.

2.2.2. Two-dimensional electrophoresis

Isoelectrofocusing was carried out using IPGphor system (Ettan IPGphor isoelectric Focusing Sistem – Healthcare). IPG gels strips, pH 3–11 NL (non linear) 13 cm, were rehydrated for 14 h, at 30 V and at 20 °C, in 250 μL of reswelling buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.1% (v/v) tertigol NP7 (Sigma, Italy),...
65 mM DTT, 0.5% (v/v) pharmalyte 3–11NL (Healthcare), tertigol NP7 (Sigma) containing 100 μg protein sample. Strips were focused at 20,000 V h, at constant temperature of 20 °C and the current was limited to 50 A per IPG gel strip. After isoelectrofocusing the strips were stored at −80 °C until use or equilibrated immediately for 10–12 min in 5 ml of equilibration buffer [50 mM Tris pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 3% (w/v) iodoacetamide (Healthcare)]. Then, the immobile IPG gel strips were applied to 15% SDS–PAGE without a stacking gel. The separation was performed at 80 V for 17 h at room temperature. The 2D gels were fixed for 2 h in fixing solution [(ethanol 40% (v/v) acetic acid 10% (v/v) (VWR International, Italy)], stained with fluorescent staining (FlamingoTM Fluorescent Gel Stain by BIO-RAD, Italy) for 3 h and destained with 0.1% (w/v) Tween 20 (VWR International) solution for 10 min. Triplicate gels of each mix sample were obtained, visualized using a Typhoon laser scanner (Healthcare) and analyzed with Platinum Software (Healthcare). The analysis was performed comparing FLUVA, ATO and FENO with CTRL. For each analysis one gel was chosen as the master gel, and used for the automatic matching of spots in the other 2D gels. Only spots present in all gels used for the analysis were considered. The software provided normalized volume for each spot (representing protein amount). A good reproducibility of the spots among the triplicate gels of each experimental group was found. Plotting the spot volumes for matched spots on a linear scale, in fact, regression analysis yielded correlation coefficients in the range 0.75–0.8. The volumes of each spots in the triplicate gels were averaged and the average volume used for statistical comparison among spots was considered significant if P < 0.05. The average volumes of each differentially expressed spot were used to determine the volume ratios reported in the figures and tables.

2.3. Protein identification

2.3.1. Electrophoresis fractionation and in situ digestion

2D gels were loaded with 300 μg of proteins per strip and the electrophoretic was carried out with the same conditions described above. After staining with Colloidal Coomassie (Thermo scientific, UK) spots were excised from the gel and washed in 50 mM ammonium bicarbonate pH 8.0 in 50% acetonitrile (VWR International) to a complete destaining. The gel pieces were re-suspended in 50 mM ammonium bicarbonate pH 8.0 containing 100 ng of trypsin (Sigma–Aldrich) and incubated for 2 h at 8 C and overnight at 37 °C. The supernatant containing the resulting peptide mixtures was concentrated and washed (i) at 1 μl/min onto a C18 reverse-phase pre-column (Waters, USA) or (ii) at 4 μl/min in 40 nl enrichment column (Agilent Technologies chip), with 0.1% formic acid (VWR International) as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75 mm × 20 cm in the Waters system, 75 mm × 43 mm in the Agilent Technologies chip) at a flow rate of 200 nl/min, with a linear gradient of eluent B [0.1% formic acid in acetonitrile (VWR International)] in A (0.1% formic acid) (VWR International) from 5% to 60% in 50 min. Elution was monitored on the mass spectrometers without any splitting device. Peptide analysis was performed using data-dependent acquisition of one MS scan (m/z ranges from 400 to 2000 Da) followed by MS/MS scans of the three most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (2 min) of ions from which definitive mass spectral data had previously been acquired. Moreover a permanent exclusion list of the most frequent peptide contaminants (keratins and trypsin peptides) was included in the acquisition method in order to focus the analyses on significant data.

2.4. Immunoblot analysis

The validation of expression changes of the proteins as judged by 2-DE analysis was carried out by standard 1-D immunoblotting. About 30 μg of muscle samples prepared and used for 2D electrophoresis were loaded on 15% polyacrylamide SDS–PAGE gels. The proteins were electrotransferred from gels to nitrocellulose membranes (4.5 μm pore size, Healthcare product, Germany) and the Western blot analysis was performed. Nitrocellulose membranes were blocked in 5% milk in TBS (Tris 0.02 M, NaCl 0.05 M pH 7.4–7.6) for 1 h and then incubated in primary antibody (diluted in 5% milk) at 4 °C overnight. The membranes were probed with antibody specific to ATP synthase (rabbit-anti ATP synthase, 1:2500, from Abcam), Pyruvate kinase (rabbit polyclonal anti pyruvate kinase, 1:1000, from Abcam), Glyceraldehyde 3-P dehydrogenase (rabbit polyclonal anti GAPDH, 1:1000, from Abcam), Triosephosphate isomerase (goat polyclonal anti Triosephosphate isomerase 1:1000, from Biocompare, Italy), Creatine Kinase M (rabbit-anti CK M, 1:1000, from Abcam) Hsp1 (mouse-anti Hsp27, 1:1000, from Abcam). After several rinses in TTBS (0.1% Tween-20 in TBS, VWR International), the membranes were incubated in HRP-conjugated secondary antibody (diluted in 5% milk), rabbit-anti-mouse (1:800, from Dako, USA), rabbit-anti-goat (1:5000, from Abcam) or goat-anti-rabbit (1:5000, from Millipore, Italy) for 1 h at room temperature. The protein bands were visualized by an enhanced chemiluminescence method in which luminol was excited by peroxidase in presence of H2O2 (ECL Plus, Healthcare product). The content of single protein investigated was assessed by determining the brightness-area product (BAP) of the protein bands.

2.5. Analysis of MHC isoforms content

About 6 μg of each muscle sample were dissolved in lysis buffer containing 8 M, 2M thiourea, 4% CHAPS, 65 mM DTT and 40 mM Tris base [36]. The lysates were applied onto 8% polyacrylamide SDS–PAGE gels prepares according to the method described by Talmadge and Roy [37]. Electrophoresis was run for 2 h at 200 V and then for 24 h at 250 V. The gels were stained with Coomassie blue staining. In the region of MHC isoforms, four bands were separated that corresponded, in order of migration from the fastest to the slowest, to MHC-1 (or slow), MHC-2B, MHC-2X and MHC-2A (or fast). Densitometric analysis of these isoforms were performed.
to establish the relative proportion of the MHC isoforms identified in the sample [38].

2.6. Creatine kinase determination

Blood was collected by cardiac puncture, soon after animal death, in ethylenediaminetetraacetic acid (EDTA, Sigma) rinsed centrifuge tubes. The blood was centrifuged at 600 x g for 10 min at 15 °C and the plasma was separated and stored at −20 °C until assay. Creatine kinase determination was performed by standard spectrophotometric analysis by using diagnostic kit (Sigma–Aldrich, Milan, Italy) within 7 days from plasma preparation [24].

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical significance of the differences between means was assessed by Student’s t-test or by one-way ANOVA followed by Student–Newman–Keuls test (for data in Fig. 3). A probability of less than 5% was considered significant (P < 0.05).

3. Results

3.1. Analysis of skeletal muscle proteomic map

Proteomic maps of EDL muscles, for each animal of each group (control rats, statin and fibrate treated rats), were obtained using 2D gel electrophoresis. The comparison between 2D maps of treated and control rats enabled us to identify the differentially expressed proteins which were thereafter identified by MALDI. Such proteins are indicated by circles and numbers in the control maps of EDL as shown in Fig. 1. The full set of information regarding the existence of isoforms or to post-translational modifications of proteins. Different isoforms of the same protein might differ both for molecular weight and isoelectric point, or different molecular weight. Mass spectrometry was not able to identify all isoforms of some proteins or the nature of their post-translational modifications. To make sure that the change in expression of the identified spot or spots of a given protein was representative of the global change of that protein, the expression of the most relevant proteins was confirmed by western blot on one dimensional gels. In the latter gels, in fact, all the spots of a protein which are separated in 2D gels migrate in a single band.

3.2. Energy production system

The larger group of differently expressed proteins was involved in the three energy production systems: oxidative metabolism, glycolytic metabolism and creatine kinase. EDL of atorvastatin treated rats showed a down regulation of four proteins involved in oxidative metabolism [isocitrate dehydrogenase 3 (−5.49 ratio), NADH dehydrogenase 1 (−1.98 ratio), oxoglutarate dehydrogenase (−1.9 ratio), ATP synthase (β subunit) (−6.43 ratio)]. The glyceraldehyde 3-phosphate dehydrogenase, enolase 3 and three isoforms of pyruvate kinase (spot number 8, 9 and 10), that are involved in glycolytic metabolism, were also down-regulated (−3.30, −3.17, −3.01, −1.377 and −2.39 ratio, respectively) (Table 1). EDL of fluvastatin treated animals did not show significant alterations of proteins involved in energy production systems, except for 2 isoforms of β enolase 3 (protein of glycolytic system) that was less expressed if compared to control (−2.20 and −1.73 ratio in spot number 1 and 2, respectively) (Table 1). In contrast, the fenofibrate maps showed small alterations of the enzymes involved in oxidative metabolisms and a marked down-regulation of proteins involved in the glycolytic metabolism. The ATP synthase, in particular the mitochondrial β subunit (−2.29 ratio), which belongs to the oxidative system, was down-regulated. Triosephosphate isomerase, β enolase 3, and 2 isoforms of glyceraldehyde 3 phosphate dehydrogenase (spot number 22 and 23), which belongs to glycolytic systems, were down-regulated (−1.91, –4.09, −2.35 and −6.08 ratio, respectively). On the contrary the pyruvate kinase was up-regulated (2.17 ratio) (Table 1). EDL of fenofibrate and atorvastatin treated rats showed a significant down-regulation of creatine kinase [−1.62 ratio for fenofibrate map (spot number 16) and −2.17, −2.90 ratio for atorvastatin map (spot number 16 and 17)] compared to CTRL.

3.3. Myofibrillar proteins

All treatments resulted in some alteration in myofibrillar protein expression. The troponin T type IVc was down-regulated in
muscles treated with fenofibrate (−1.84 ratio), and fluvastatin (−1.71). The troponin T fast and α-2 actin were down-regulated following fenofibrate treatment (−3.21 and −1.95, respectively). In fluvastatin treated muscles myozenin 1 was down regulated (−1.64 ratio). The troponin T fast was down-regulated following atorvastatin treatment (−2.29 ratio) (Table 1). The adaptations in myofibrillar protein isoforms, consistent with the lack of variation in MHC isoform composition, did not show any trend of a shift in muscle phenotype (Fig. 3).

3.4. Detoxification system

Statin and fibrate had an impact on detoxification and on antioxidant proteins. The heat shock protein 1 was, indeed, down-regulated after treatment with atorvastatin and fenofibrate (−3.26 and −1.85 ratio, respectively). The carbonic anhydrase III was under-expressed after fluvastatin and atorvastatin treatments (−1.78 ratio and −2.66, respectively). The map of EDL muscle treated with fluvastatin showed an overexpression of chaperonin 60 (1.57 ratio) (Table 1).

3.5. Other proteins

The atorvastatin map showed a down regulation of serum albumin, a known plasma protein carrier (−2.03 ratio). The muscles treated with fenofibrate showed an important reduction of the expression of MSF mitochondrial factor, an important stimulation factor (−6.62 ratio) (Table 1).
3.6. Immunoblot analysis

To validate the results of 2-DE analysis comparative immunoblotting of some of most relevant changed proteins was carried out. Comparative analysis of immunoreactive bands of ATP synthase, isocitrate dehydrogenase, pyruvate kinase, glyceraldehyde 3-P dehydrogenase, triosephosphate isomerase, creatine kinase, Hsp1 in the different treatments, confirmed, at the level of the whole protein, the change in expression of the protein spot/s revealed by 2D gels (Fig. 4).

3.7. Biological and functional indexes of skeletal muscle toxicity

To verify the possible impairment of skeletal muscle due to statin or fibrate chronic treatment we examined the release of creatine kinase (CK) from myocytes into the bloodstream. Thus, we measured CK level in plasma of all treated rats and compared those with control. Fluvastatin at the higher dose (20 mg/kg) significantly increased (about 3-fold) the plasma CK content, while no significant effect was found in the rats treated with the lower dose (Table 2). Also atorvastatin and fenofibrate, at the doses tested, significantly increased plasma CK level about 3-fold or 2-fold, respectively, with respect to control (Table 2). The histopathological analysis of tibialis muscle did not show any modification of muscle section (data not shown), as previously described [24]. Moreover, as already demonstrated [24], in all the rats treated with fluvastatin, atorvastatin or fenofibrate the resting gCl was significantly reduced (no more than 30%) with respect to the control value (data not shown).

4. Discussion

Taking advantage of the high resolving power and of the very large number of proteins sampled by the proteomic analysis, this novel study demonstrates that statins and fibrate administration induce complex modifications of the protein pattern of skeletal muscle, suggesting an effect on a wide range of biological functions (Supplementary Fig. S1). In parallel, other studies performed by using combined genomic and lipidomic analysis showed changes in muscle metabolism (dysregulation of calcium binding proteins and phospholipase C pathway, defective mitochondrial metabolism and activation of pro-apoptosis pathway, dysregulation of cell membrane lipids) contributing to muscle toxicity [39]. Our results showed a down-regulation of several muscle proteins and this effect was confirmed by the western blot experiments. Proteins involved in energy production and in detoxification systems were the major sets of differentially expressed proteins and their changes could lead to the generation of myopathy. Atorvastatin induced a modification of a larger number of proteins than fluvastatin and fenofibrate. The reason may be related to the different chemical structure or the longer half-life of atorvastatin in comparison to the other drugs tested.

4.1. Myofibrillar proteins

Muscle plasticity is a particular feature of skeletal muscle by which different stimuli (disuse, drugs, etc.) can induce phenotype transition. For instance, hindlimb unloading of rodents, a model of muscle disuse, induce a slow-to-fast transition of postural muscles, i.e. increased expression of fast isoforms of myosin heavy chain (MHC) and increase of resting gCl in soleus muscle [40]. A coordinated expression existed among MHC isoforms, metabolic enzymes and isoforms of other myofibrillar and non-myofibrillar proteins [41–43]. Therefore, in most myopathic conditions the change in expression of many proteins can be related to the activation of a single gene expression program aimed to coordinately adapt muscle contractile machinery, energy production and cytoskeleton. To test if statin or fibrate can produce phenotype transition, we measured MHC isoform distribution in the EDL muscle. The lack of any change in MHC isoform distribution indicates that the large changes in the protein pattern due to statin and fibrate administration occur independently from...
such major gene expression program, suggesting more a toxic action than a functional adaptation of the treatments. This observation especially applies to changes in energy production systems which are generally under the same control as MHCs, but there is a likelihood slight effect on the oxidative metabolism were observed from muscle phenotype.

4.2. Oxidative energy metabolism

Both statins and fenofibrate induced critical alterations of energy metabolism enzymes: atorvastatin down-regulated different oxidative and glycolytic enzymes, whereas fenofibrate mainly affected glycolytic enzymes. This latter finding can be a consequence of the inhibition of the specific targets of the two drugs. Otherwise, the prevalent effect of atorvastatin on the oxidative metabolism pathway can be related to the already described statin-mediated mitochondrial involvement [17,19,39]. Yet, since fenofibrate have no effects on calcium released by mitochondria [17], likely slight effect on the oxidative metabolism were observed here.

4.2.1. Oxidative metabolism

Two biochemical pathways part of the oxidative metabolism, such as the Krebs cycle and the mitochondrial respiratory chain, are affected by atorvastatin treatment. Indeed two enzymes that participate to Krebs cycle, such as isocitrate dehydrogenase (which

Table 1

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Accession num.</th>
<th>Fold change</th>
<th>MOWSE</th>
<th>P value</th>
<th>Th Pi</th>
<th>Th MW</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enolase 3</td>
<td>gi126723393</td>
<td>-2.20323</td>
<td>90</td>
<td>0.0491</td>
<td>7.08</td>
<td>47,326</td>
<td>Glycolitic metabolism</td>
</tr>
<tr>
<td>2</td>
<td>Enolase 3</td>
<td>gi126723393</td>
<td>-1.7359</td>
<td>412</td>
<td>0.039</td>
<td>7.08</td>
<td>47,326</td>
<td>Glycolitic metabolism</td>
</tr>
<tr>
<td>3</td>
<td>Troponin T class IVC beta-2</td>
<td>gi207403</td>
<td>-1.711174</td>
<td>383</td>
<td>0.0258</td>
<td>9.36</td>
<td>28,595</td>
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</tr>
<tr>
<td>4</td>
<td>Myozenin 1</td>
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<td>8.57</td>
<td>31,774</td>
<td>Myofibrillar systems</td>
</tr>
<tr>
<td>5</td>
<td>Carbonic anhydrase III</td>
<td>gi31377484</td>
<td>-1.78059</td>
<td>497</td>
<td>0.0203</td>
<td>6.89</td>
<td>29,698</td>
<td>Detoxification systems</td>
</tr>
<tr>
<td>6</td>
<td>Chaperonin 60</td>
<td>gi1778213</td>
<td>1.57865</td>
<td>389</td>
<td>0.0492</td>
<td>5.78</td>
<td>61,029</td>
<td>Detoxification systems</td>
</tr>
</tbody>
</table>

| 7        | Isocitrate dehydrogenase 3 (NAD+) alpha precursor | gi16758446 | -5.49956    | 221   | 0.0014  | 6.47  | 40,044| Oxidative metabolism |
| 8        | Pyruvate kinase | gi16757994 | -3.01587    | 870   | 0.0023  | 6.63  | 584   | Glycolitic metabolism |
| 9        | Pyruvate kinase | gi16757994 | -1.37775    | 976   | 0.033   | 6.63  | 58,294| Glycolitic metabolism |
| 10       | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10-like | gi32996721 | -1.98637    | 309   | 0.0172  | 7.14  | 40,804| Oxidative metabolism |
| 12       | ATP synthase subunit beta, mitochondrial | gi1374715  | -6.43293    | 510   | 0.0012  | 4.95  | 51,710| Oxidative metabolism |
| 13       | Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) precursor | gi62945278 | -1.90863    | 333   | 0.0298  | 6.3   | 117,419| Oxidative metabolism |
| 14       | Glyceraldehyde 3-phosphate dehydrogenase | gi56188    | -3.3096     | 316   | 0.0076  | 8.43  | 36,998| Glycolitic metabolism |
| 15       | Enolase 3    | gi126723393    | -3.17        | 412   | 0.047   | 7.08  | 47,326| Glycolitic metabolism |
| 16       | Fast skeletal muscle troponin T | gi136385   | -2.29586   | 267   | 0.019   | 6.19  | 30,732| Myofibrillar systems |
| 17       | Creatine kinase | gi6978661 | -2.17813    | 530   | 0.0043  | 6.58  | 43,220| Energy production system |
| 18       | Creatine kinase | gi6978661 | -2.93083    | 480   | 0.019   | 6.58  | 43,220| Energy production system |
| 19       | Fast skeletal muscle troponin T | gi31377484 | -2.66095    | 375   | 0.0032  | 6.89  | 29,698| Detoxification system |
| 20       | Heat shock protein 1 | gi94400790 | -3.26578    | 265   | 0.0024  | 6.12  | 24,860| Detoxification system |
| 21       | Serum albumin | gi124028612  | -2.03454    | 162   | 0.0361  | 6.09  | 70,682| Transport protein |

In the table are showed: the spot number corresponding to the number reported in Fig. 2; the name of proteins; the accession number corresponding to NCBI; the related fold changes of proteins are expressed in average ratio of change relative to the control (V Treated/V ctrl); MOWSE score; P value; the experimental Pi; the experimental MW; the biochemical pathway group membership.

Fig. 3. Myosin heavy chain (MHC) isoform distribution in EDL muscles of rats following treatment with statins or fenofibrate.
converts isocitrate in oxoglutarate) and the oxoglutarate dehydrogenase (which converts ketoglutarate in succinyl CoA) are downregulated. Both enzymes are NAD dependent and their downregulation can affect NADH production, then blocking the pathway and ATP production during mitochondrial electron transport. In consequence the acetyl CoA may accumulate in the mitochondria and in the cytosol, because it is the first substrate of cholesterol synthesis that is inhibited by the

Fig. 4. Confirmation of the proteomic data by Western blot analysis. The figure shows immunoreactive bands of ATP synthase, isocitrate dehydrogenase, pyruvate kinase, glyceraldehyde 3-P dehydrogenase, triosephosphate isomerase, creatine kinase, Hsp1 and their quantification by the levels of brightness area product (BAP) in control (CTRL) mice and mice treated with atorvastatin (ATO) and fenofibrate (FENO).
statin. Increase of acetyl-CoA is known to inhibit the pyruvate dehydrogenase (PK) and to slow the glycolysis. The enzymes NADH dehydrogenase (ubiquinone) and ATP synthase, involved in the mitochondrial respiratory chain, were also altered by atorvastatin treatment. As already suggested, ubiquinone reduction may be responsible for statin induced muscle damage [19]. For instance it has been shown that simvastatin was able to reduce ubiquinone level and mitochondrial function in human skeletal muscle [44]. Although controversial data have been reported [45,46], it has proposed that muscle cell treatment with ubiquinone can restore statin-induced muscle damage [47]. Thus the observation that NADH dehydrogenase was down-regulated in the proteomic map of atorvastatin treated skeletal muscles strongly support the hypothesis of mitochondrial involvement [17]. Another novelty of the present study is the down-regulation and defect of the catalytic subunit of ATP synthase due to atorvastatin treatment, which may lead to ATP level reduction and consequent metabolic stress. Accordingly, a reduction of intracellular ATP content has been already described in human myocytes due to atorvastatin [48]. Here we also find a minor involvement of ATP synthase in fenofibrate treated rats supporting the lesser participation of mitochondria in skeletal muscle damage induced by fenofibrate [17].

4.2.2. Glycolytic metabolism

Both fenofibrate and statins resulted in a down-regulation of glycolytic enzymes expression. The beta enolase was down-regulated in all treatments, the PK was down-regulated in rats treated with atorvastatin and glyceraldehyde-3-phosphate dehydrogenase was down-regulated following atorvastatin and fenofibrate treatments. Another glycolytic enzyme, such as the triose-phosphate isomerase, essential for energy production, was down-regulated after fenofibrate treatment. The slow-down of glycolysis can be justified by the increased β-oxidation pharmacologically induced by PPAR mediated fibrate or statin activation [49]. The down-regulation of glycolytic enzymes is a symptom of energy production failure, and can contribute to muscle damage. Interestingly a general impairment of energy metabolism has been observed in conditions of muscle atrophy [29], which may develop also during statin myopathy [50]. Moreover, it has been shown that hereditary muscle glycogenoses in humans are characterized by defective glycolytic enzymes, including beta enolase, and leads to different degree of myopathy, ranging from cramps to myoglobinuria [51,52]. The PK is a key enzyme in the glycolytic pathway, being responsible for the synthesis of pyruvate and ATP formation; it is allosterically inhibited by the increase in acetyl CoA, which in this condition may be induced by the block of cholesterol pathway in the cytosol. Interestingly, in fenofibrate treated rats the PK is up-regulated, although many enzymes of the glycolytic pathway are down-regulated, possibly because the oxidative and cholesterol pathway are not affected.

4.3. Energy production system: creatine kinase

Muscle damage induced by statin and fibrate is associated with an increase of creatine kinase (CK) level in the plasma. For instance, other authors found a plasma CK increase in correlation with histological damage due to cerivastatin induced skeletal muscle damage [53]. Here we found an increase of plasma CK in all treated rats not followed by histological modification likely because the drugs we used were less toxic. Moreover, we found a decrease of CK expression in muscle treated with atorvastatin and fenofibrate. Since phosphocreatine, produced by CK, is an energy reservoir for different tissues and especially for skeletal muscle, the decrease in CK, together with the observed down-regulation of oxidative and glycolytic enzymes, suggest a general impairment of energy metabolism, which in turn could decrease protein synthesis, an ATP-requiring process. Interestingly, it has been shown that in transgenic mice lacking either the cytoplasmic or mitochondrial CK, muscle atrophy and damage occur [54]. Surprisingly, no modification of CK expression was found in fluvastatin treated rats, in accord with the minor impact of this drug of the whole proteomic pattern.

4.4. Detoxification systems

Statin administration and, to some extent, fibrate administration down-regulated detoxification systems: heat shock protein 1, which protects cells against free radicals produced by stress stimuli, as well as carbonic anhydrase that protects cells against oxidative damage by binding free radicals [55,56]. An effect of statin on HSPs expression has been previously observed in other tissues, such as human placental cells line [57] and rat retina during ischemia [58]. Together with the decrease of muscle plasmalogens, a sub-class of ether phospholipids involved in protection against oxidative damage [39], these effects suggest an accentuated sensitivity of statin-treated muscles to oxidative stress. Oxidative stress has been pinpointed as a likely trigger of many conditions of muscle wasting [59,60].

4.5. Other proteins

Although our previous studies have demonstrated no modification of mitochondrial activity in fibrate-induced myopathy [17], here we found a down-regulation of the mitochondrial stimulation factor (MSF) important for the synthesis and folding of mitochondrial ATP-dependent protein precursor [61], likely suggesting an alteration in the number of these organelles in fenofibrate-treated muscles. We also found a down-regulation of serum albumin in atorvastatin-treated muscles likely supporting the lack of its scavenger activity in the muscle. At this regard the effects of statin on albumin expression is controversial: simvastatin has been found to increase albumin synthesis contributing to reduce cardiovascular risk since it transport plasma lipids [62].

5. Concluding remarks

By using a proteomic approach we identified new toxicological markers of myopathy induced by hypolipidemic drugs (Supplementary Fig. S1). The major observations of this study are the following: (1) in line with our previous studies [20] atorvastatin was found to affect the proteomic profile of the EDL muscle more deeply than fluvastatin, likely due to its structural or pharmacokinetic characteristics; (2) atorvastatin mainly impaired the metabolic pathways responsible for energy production and particularly the key enzymes of the mitochondria, while fenofibrate essentially affected the glycolytic metabolism. Such a perturbation in energy metabolism and ATP synthesis may have

**Table 2**

Effects of two months chronic treatment with statins or fenofibrate on rat plasma creatine kinase.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>CK (U/l)</th>
<th>Bonferroni t-test vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>863 ± 210</td>
<td></td>
</tr>
<tr>
<td>Fluvastatin 5 mg/kg</td>
<td>8</td>
<td>1011 ± 199</td>
<td>ns</td>
</tr>
<tr>
<td>Fluvastatin 20 mg/kg</td>
<td>8</td>
<td>2358 ± 250</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Atorvastatin 10 mg/kg</td>
<td>8</td>
<td>2561 ± 244</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Fenofibrate 60 mg/kg</td>
<td>8</td>
<td>1844 ± 217</td>
<td>P &lt; 0.005</td>
</tr>
</tbody>
</table>

The values of creatine kinase (CK) measured in plasma of control and treated rats are expressed as mean ± S.E.M. from the number (N) of animals as indicated. Data showed significant differences by ANOVA test (F = 11.7, df = 4/35, P < 0.001) followed by Bonferroni t-test.
a profound impact on protein synthesis and cell viability [63]; (3) considering the pivotal role attributed to oxidative stress in many myopathies, the impairment of detoxification systems could play a major role in the development of myopathy; (4) since an impaired glycolytic metabolism was observed without alteration of MHC expression, the effects of statin and fenofibrate are independent of phenotype-specific gene reprogramming. This work adds important novel information regarding the molecular and functional mechanism of drug-induced muscle damage and opens the way to the discovery of new drug targets to counteract muscle toxicity.

Conflicts of interest
The authors declare no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.01.022.

References


