

Effects of low and high doses of fenofibrate on protein, amino acid, and energy metabolism in rat

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Summary

A feared adverse effect of dyslipidaemia therapy by fibrates is myopathy. We examined the effect of fenofibrate (FF) on protein and amino acid metabolism. Rats received a low (50 mg/kg, LFFD) or high (300 mg/kg, HFFD) dose of FF or vehicle daily by oral gavage. Blood plasma, liver, and soleus and extensor digitorum longus muscles were analysed after 10 days. The FF-treated rats developed hepatomegaly associated with increased hepatic carnitine and ATP and AMP concentrations, decreased protein breakdown, and decreased concentrations of DNA and triglycerides. HFFD increased plasma ALT and AST activities. The weight and protein content of muscles in the HFFD group were lower compared with controls. In muscles of the LFFD group there were increased ATP and decreased AMP concentrations; in the HFFD group AMP was increased. In both FF-treated groups there were increased glycine, phenylalanine, and citrulline and decreased arginine and branched-chain keto acids (BCKA) in blood plasma. After HFFD there were decreased levels of branched-chain amino acids (BCAA; valine, leucine and isoleucine), methionine, and lysine and increased homocysteine. Decreased arginine and increased glycine concentrations were found in both muscles in FF-treated animals; in HFFD-treated animals lysine, methionine, and BCAA were decreased. We conclude that FF exerts protein-anabolic effects on the liver and catabolic effects on muscles. HFFD causes signs of hepatotoxicity, impairs energy and protein balance in muscles, and decreases BCAA, methionine, and lysine. It is suggested that increased glycine and decreased lysine and methionine levels are due to activated carnitine synthesis; decreased BCAA and BCKA levels are due to increased BCAA oxidation.

KEYWORDS

branched-chain amino acids, carnitine, fibrates, hepatomegaly, methionine

1 | INTRODUCTION

Fenofibrate (FF) is a drug of the fibrate class used widely to treat dyslipidaemia. Fibrates reduce cholesterol and triglyceride levels and thus decrease risk for cardiovascular diseases. The mechanism of fibrate action is known to be mediated by activation

of peroxisome proliferator-activated receptor alpha (PPAR α), a transcriptional factor that is abundantly expressed in the liver. Upon agonist binding, PPAR α stimulates expression of various genes involved in fatty acid oxidation and metabolism of lipoproteins. The well-known pharmacological effects are activated carnitine synthesis, peroxisome proliferation, and accelerated

β -oxidation. Fibrates exert pleiotropic effects, and beyond lowering blood lipids affect microcirculation, oxidative stress, and triglyceride content in the liver.¹⁻³ It is supposed that their use may have benefits in adults with inflammatory disorders, and in the elderly, and improve liver function tests in patients with hepatic steatosis, in addition to patients with clinical dyslipidaemia.^{2,3}

In general, fibrates are well tolerated, and side effects are rare.⁴ The most feared adverse effect of the clinical use of fibrates is myopathy, evidenced by muscle weakness and/or pain and increased serum levels of creatine phosphokinase. Histological examinations show scattered muscle fibre necrosis. Withdrawal of the drug is usually followed by rapid improvement of the symptoms; therefore, severe cases of life-threatening rhabdomyolysis are rare.⁵⁻⁷

The pathogenesis of fibrate-induced myopathy is unclear. The studies report perturbations in energy metabolism homeostasis, mitochondrial dysfunction, and lactic acidosis.^{8,9} Impaired glutathione biosynthesis and enhanced generation of reactive oxygen species markers indicate the possibility of oxidative stress.⁹ Several studies have shown that fibrates decrease the levels of branched-chain amino acids (BCAA; valine, leucine and isoleucine),^{10,11} essential amino acids with protein-anabolic properties.

To better understand the effects of fibrates on skeletal muscle, a study has been performed using male rats. We have examined two different FF doses: the low dose (LFFD, 50 mg/kg body weight), which represents the usual situation in human therapy, and the high dose (HFFD, 300 mg/kg body weight), which induced adverse effects in some experimental studies.^{9,12,13} The focus of our study was on alterations in protein and amino acid metabolism. By examining alterations in the level of adenine nucleotides, we tried to estimate the effects of fibrates on energy metabolism. Because there are different sensitivities of slow- and fast-twitch muscles to various signals,¹⁴⁻¹⁶ muscles of different metabolic properties were examined.

2 | MATERIALS AND METHODS

2.1 | Animals and material

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing approximately 240 g were housed in standardized cages in quarters with controlled temperature and a 12-hour light-dark cycle. Rats were maintained on an ST-1 (Velas, CR) standard laboratory diet containing (w/w) 24% nitrogenous compounds, 4% fat, 70% carbohydrates, and 2% minerals and vitamins, and were provided drinking water ad libitum.

Fenofibrate (Fenofix) was obtained from Ingest Industrial Solutions (Brno, Czech Republic). The chemicals were

purchased from Sigma-Aldrich, Lachema, Waters, Biomol and Merck.

2.2 | Ethical approval

The Animal Care and Use Committee of Charles University, Faculty of Medicine in Hradec Kr.lov. (licence no.144879/2011-MZE-17214), specifically approved this study on 1 November 2016 (identification code MSMT-33747/2016-3). All experimental procedures complied with the National Institutes of Health guidelines.

2.3 | Experimental design and methods

The rats ($n = 25$) were randomly divided into three groups to receive daily oral gavage of FF dissolved in water for 10 days as follows: low dose of fenofibrate (LFFD group; 50 mg/kg body weight, $n = 9$), high dose of fenofibrate (HFFD group; 300 mg/kg body weight, $n = 8$), or the same volume of vehicle (control group, $n = 8$). The doses were selected according to those used to examine the therapeutic effects of fibrates in rats.^{9,12,13}

At the end of the study, the overnight fasted animals were euthanized in ether anaesthesia by exsanguination from the abdominal aorta. Blood was collected in heparinized tubes and immediately centrifuged for 15 minutes at 2200 g using a refrigerated centrifuge; blood plasma was transferred into polypropylene tubes using a Pasteur pipette. The liver and soleus (SOL, red, slow-twitch muscle) and extensor digitorum longus (EDL, white, fast-twitch muscle) muscles were quickly removed and weighed. Small pieces (~0.1 g) of the tissues were frozen in liquid nitrogen.

2.4 | Biochemical markers in blood plasma, liver and muscles

Plasma levels of glucose, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides and cholesterol were measured using commercial tests (Boehringer, Mannheim, Germany; Elitech, Sées, France, Sigma Chemical, St. Louis, MO, USA, and Roche Diagnostics, Basel, Switzerland). The DNA content in the liver was determined using the diphenylamine reaction.¹⁷ Protein contents in the liver and muscles were measured according to Lowry et al.¹⁸ Concentration of triglycerides in the liver was determined by ethanolic potassium hydroxide saponification followed by an assay for glycerol (Sigma Chemical). Carnitine (β -hydroxy- γ -N-trimethylaminobutyric acid) concentration in blood plasma and liver was measured enzymatically as described by Alhomida et al.¹⁹

2.5 | Chymotrypsin-like activity (CHTLA) of proteasome and cathepsin B and L activities in liver and muscles

The CHTLA of the proteasome and cathepsin B and L activities were determined using the fluorogenic substrates Suc-LLVY-MCA,²⁰ and Z-FA-MCA,²¹ respectively, as previously described in detail.²² The fluorescence of the samples was measured at the excitation wavelength of 340 nm and the emission wavelength of 440 nm (Tecan Infinite™ 200). A standard curve was established for 7-amino-4-methylcoumarin (AMC), which allowed expression of the enzyme activities in nmol of AMC/mg protein/h.

2.6 | Amino acid concentrations

Amino acid concentrations were determined in supernatants of deproteinized samples of blood plasma, liver and muscles by high-performance liquid chromatography (HPLC; Alliance 2695, Waters) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters) using norleucine as the internal standard. The level of total homocysteine in plasma was determined by enzymatic conversion of homocysteine to S-adenosyl-L-homocysteine, followed by quantification of S-adenosyl-L-homocysteine by an enzyme-linked immunoassay (ELISA) using the IMMULITE® 2000 Immunoassay System (Siemens GmbH). The results are expressed as $\mu\text{mol/L}$ of blood plasma or nmol/g of wet tissue.

2.7 | Adenine nucleotides

The reversed-phase HPLC (Alliance 2695, Waters) combined with ultraviolet detection was used for the determination of ATP, ADP and AMP in the liver and muscles as described in detail previously.²³ The results are expressed as $\mu\text{mol/g}$ of wet tissue.

2.8 | Branched-chain keto acid (BCKA) concentrations

The reversed-phase HPLC combined with o-phenylenediamine derivatization was used for the determination of BCKA in samples of blood plasma as previously described in detail.²³ The quinoxalinol derivatives of BCKAs were detected using fluorescence with emission and excitation at 410 nm and 350 nm, respectively, and were quantified by the internal standard method. The results are expressed as $\mu\text{mol/L}$ of blood plasma.

2.9 | Statistical analysis

The results are expressed as mean \pm standard error (SE). Analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc analysis was used to detect differences between multiple independent groups. NCSS 2001 statistical software (Kaysville, UT, USA) was used for analyses. Differences were considered significant at $P < .05$.

TABLE 1 Effect of fenofibrate on blood biochemistry

Variable	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
Glucose (mmol/L)	6.43 \pm 0.39	9.29 \pm 0.10*	7.09 \pm 0.15#
Creatinine ($\mu\text{mol/L}$)	23.60 \pm 1.18	16.44 \pm 0.63*	23.80 \pm 0.49#
ALT ($\mu\text{kat/L}$)	0.83 \pm 0.06	0.74 \pm 0.05	1.27 \pm 0.12*,#
AST ($\mu\text{kat/L}$)	1.46 \pm 0.07	0.86 \pm 0.11	4.23 \pm 0.68*,#
Triglycerides (mmol/L)	1.13 \pm 0.06	0.86 \pm 0.10*	0.88 \pm 0.04*
Total cholesterol (mmol/L)	1.89 \pm 0.08	1.37 \pm 0.08*	1.37 \pm 0.06*
HDL cholesterol (mmol/L)	1.13 \pm 0.07	0.74 \pm 0.05*	0.65 \pm 0.05*
LDL cholesterol (mmol/L)	0.36 \pm 0.02	0.40 \pm 0.02	0.29 \pm 0.01*,#
Non-HDL cholesterol (mmol/L)	0.76 \pm 0.06	0.63 \pm 0.03	0.72 \pm 0.06
Carnitine ($\mu\text{mol/L}$)	21.2 \pm 3.0	24.2 \pm 2.9	25.5 \pm 4.1

Note: Means \pm SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

Non-HDL cholesterol was calculated as total cholesterol – HDL cholesterol.

*Comparison vs control.

#HFFD vs LFFD.

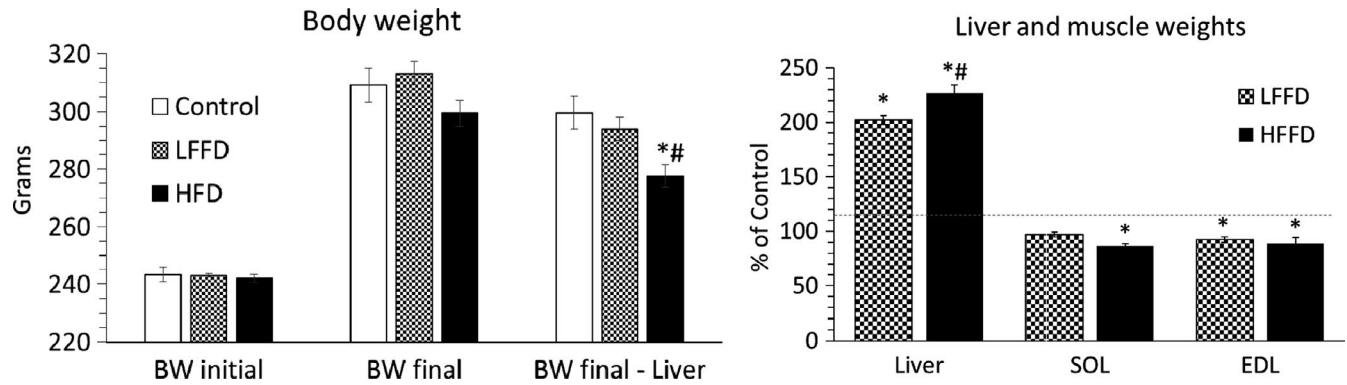


FIGURE 1 Effect of fenofibrate on body weight and weights of the liver and muscles. Means \pm SE, $P < .05$. ANOVA and Bonferroni multiple comparisons. *Comparison vs control; #HFFD vs LFFD

Variable	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
LIVER			
Weight -g	9.57 \pm 0.18	19.31 \pm 0.37*	21.73 \pm 0.72* [#]
Protein -mg/g	191 \pm 6	205 \pm 6	207 \pm 5
-g	1.82 \pm 0.05	3.96 \pm 0.17*	4.51 \pm 0.18* [#]
DNA -mg/g	1.68 \pm 0.04	1.17 \pm 0.02*	1.05 \pm 0.02* [#]
-mg	16.04 \pm 0.39	22.62 \pm 0.33*	22.78 \pm 0.86*
Triglycerides -mg/g	4.11 \pm 0.46	3.04 \pm 0.32	2.50 \pm 0.13*
-g	39.20 \pm 4.06	58.76 \pm 6.21*	54.01 \pm 2.78*
Cathepsins B and L	542 \pm 23	596 \pm 17	564 \pm 29
CHTLA	3.10 \pm 0.12	2.60 \pm 0.10*	2.61 \pm 0.14*
SOL			
Weight -mg	159 \pm 4	154 \pm 4	137 \pm 5* [#]
Protein -mg/g	142 \pm 4	130 \pm 5	138 \pm 4
-mg	22.5 \pm 0.5	20.1 \pm 1.2	18.9 \pm 0.6*
Cathepsins B and L	52 \pm 5	51 \pm 4	67 \pm 12
CHTLA	1.35 \pm 0.07	1.39 \pm 0.05	2.26 \pm 0.14* [#]
EDL			
Weight -mg	142 \pm 3	131 \pm 3*	129 \pm 3*
Protein -mg/g	140 \pm 4	139 \pm 4	139 \pm 5
-mg	19.9 \pm 0.6	18.2 \pm 0.6	17.4 \pm 0.5*
Cathepsins B and L	45 \pm 3	37 \pm 5	38 \pm 5
CHTLA	0.94 \pm 0.09	1.07 \pm 0.06	0.79 \pm 0.04

Note: Means \pm SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

The activities of cathepsins B and L and CHTLA are presented in nmol AMC/mg protein/h.

*Comparison vs control.

[#]HFFD vs LFFD.

TABLE 2 Effects of fenofibrate in the liver and muscles

3 | RESULTS

3.1 | Blood biochemical markers

Administration of LFFD increased concentrations of glucose and decreased concentrations of creatinine, triglycerides, and

total and HDL cholesterol (Table 1). HFFD decreased concentrations of triglycerides, total cholesterol, and HDL and LDL cholesterol, and increased ALT and AST levels when compared with controls. Comparison of HFFD and LFFD groups showed lower concentrations of glucose and LDL cholesterol and higher levels of creatinine, ALT, and AST in

FIGURE 2 Effect of fenofibrate on carnitine content in the liver. Means \pm SE, $P < .05$. ANOVA and Bonferroni multiple comparisons. *Comparison vs control; #HFFD vs LFFD

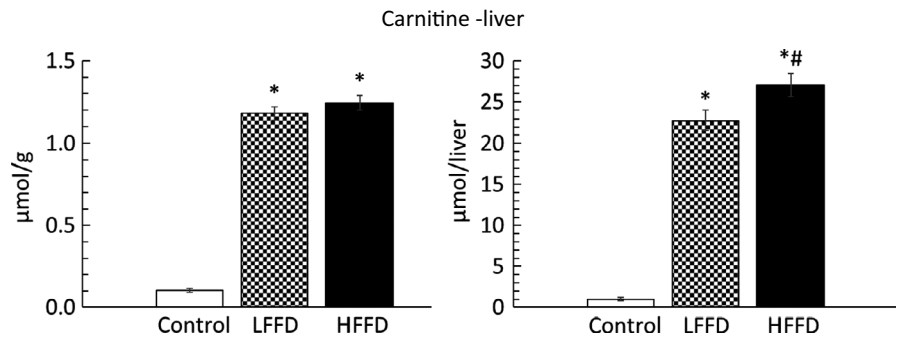
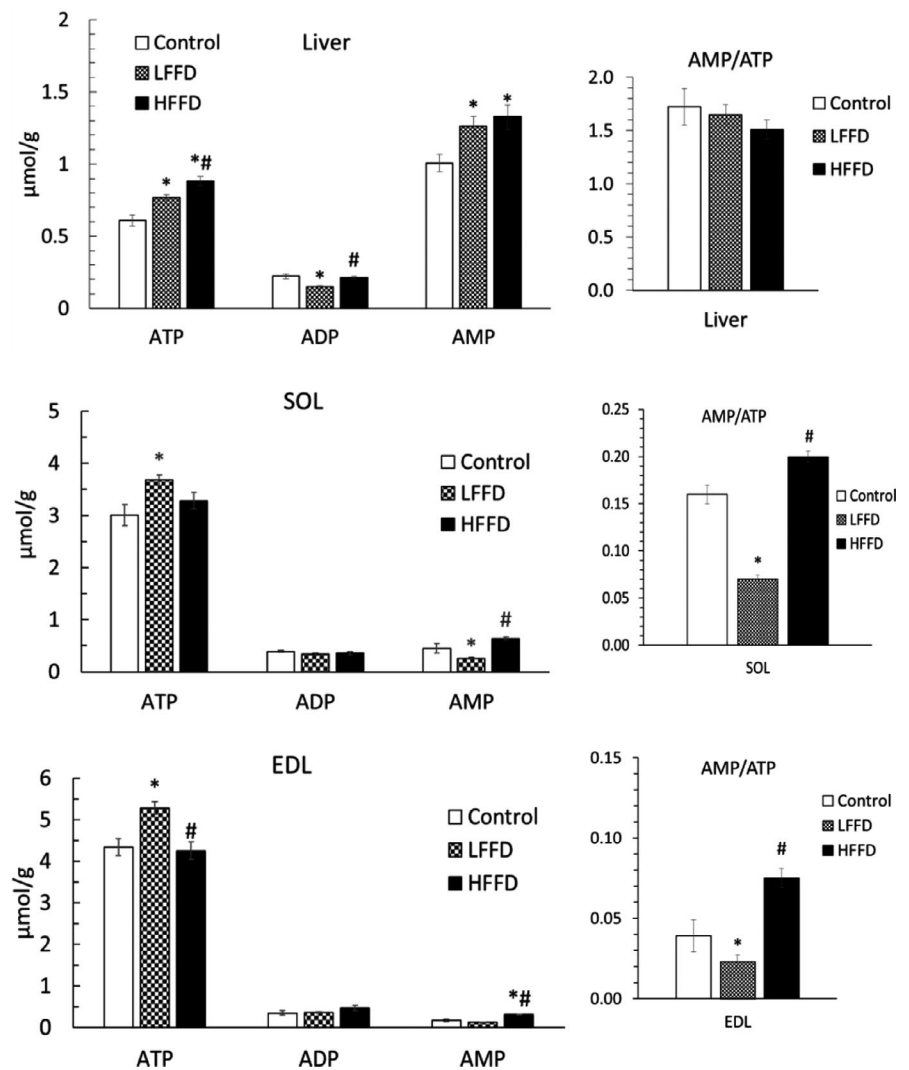


FIGURE 3 Effect of fenofibrate on adenine nucleotide concentrations and AMP-to-ATP ratios. Means \pm SE, $P < .05$. ANOVA and Bonferroni multiple comparisons. *Comparison vs control; #HFFD vs LFFD



the HFFD group. The effect of FF on carnitine concentration in blood plasma was insignificant.

3.2 | Body weight and weights of the liver and muscles

There were no significant differences among body weights of animals in specific experimental groups on the first and the last day of the experiment. At the time of euthanasia

the liver mass was 100% greater in the LFFD group and 127% greater in the HFFD group when compared with controls. After subtraction of the liver weight from the body weight, the weights of animals in the HFFD group were significantly lower when compared with the control and LFFD groups. The weights of both muscles obtained from animals in the HFFD group were significantly lower when compared with controls. In the LFFD group, a decrease in muscle mass was observed in the EDL (Figure 1, Table 2).

TABLE 3 Effect of fenofibrate on amino acid and BCKA concentrations in blood plasma

Plasma	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
EAA			
Histidine	58 ± 2	69 ± 2*	71 ± 2*
Isoleucine	113 ± 7	114 ± 4	96 ± 3*,#
Leucine	158 ± 8	177 ± 5	151 ± 7#
Lysine	447 ± 13	401 ± 16	377 ± 27*
Methionine	52 ± 2	52 ± 2	43 ± 2*,#
Phenylalanine	69 ± 2	84 ± 2*	79 ± 3*
Threonine	259 ± 16	285 ± 17	260 ± 9
Valine	198 ± 11	211 ± 5	174 ± 6#
BCAA	469 ± 25	501 ± 14	420 ± 16#
Σ EAA	1356 ± 57	1393 ± 32	1251 ± 45
NEAA			
Alanine	392 ± 19	328 ± 21	248 ± 17*,#
Arginine	162 ± 6	70 ± 4*	59 ± 3*
Asparagine	63 ± 2	58 ± 2	54 ± 4
Aspartate	12 ± 1	14 ± 1	8 ± 1*,#
Citrulline	95 ± 6	127 ± 8*	123 ± 7*
Glutamate	106 ± 5	142 ± 4*	84 ± 5*,#
Glutamine	527 ± 13	578 ± 22	627 ± 32*
Glycine	390 ± 18	677 ± 22*	712 ± 29*
Homocysteine	9.86 ± 0.77	12.90 ± 0.74	13.69 ± 1.39*
Ornithine	52 ± 3	77 ± 5*	67 ± 4*
Proline	154 ± 5	129 ± 5*	117 ± 4*
Serine	236 ± 7	277 ± 11*	258 ± 15
Taurine	311 ± 20	290 ± 25	279 ± 18
Tyrosine	110 ± 6	108 ± 4	96 ± 4
Σ NEAA	2611 ± 73	2875 ± 55*	2734 ± 89
Σ Amino acids	3967 ± 126	4268 ± 80*	3984 ± 131
KIV	10.4 ± 0.5	8.9 ± 0.2*	7.5 ± 0.4*,#
KIC	27.4 ± 1.4	24.0 ± 0.9	18.3 ± 1.2*,#
KMV	19.6 ± 0.9	16.1 ± 0.4*	13.1 ± 0.6*,#
Σ BCKA	57.4 ± 2.7	49.1 ± 1.5*	38.9 ± 2.1*,#

Note: The values are in μmol/L of plasma. Means ± SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids; KIV, α-ketoisovalerate; KIC, α-ketoisocaproate; KMV, α-keto-β-methylvalerate.

*Comparison vs control.

#HFFD vs LFFD.

3.3 | Alterations in the liver and muscles

FF had no effect on hepatic protein concentration, but it decreased DNA and triglyceride concentrations (Table 2). Due to a marked increase in the liver weight, the contents of proteins, DNA and triglycerides were higher in FF-treated animals compared with controls.

There were no differences in muscle protein concentration among experimental groups. Due to reduced muscle mass, the protein content decreased in the muscles of HFFD-treated animals.

A statistically significant decrease in hepatic CHTLA, indicating decreased protein breakdown, was observed in both groups of FF-treated animals. Cathepsin B and L activities were not affected. The effect of FF on proteolysis in muscles was mostly insignificant; the exception was an increase in CHTLA in SOL of the HFFD-treated group.

3.4 | Hepatic carnitine concentration

In the liver of FF-treated animals, carnitine concentration and carnitine content were markedly increased (23- and 27-fold after LFFD and HFFD, respectively) (Figure 2).

3.5 | Adenine nucleotide concentrations

ATP and AMP concentrations in the liver were higher in FF-treated rats when compared with controls (Figure 3). In muscles of the LFFD group, we found increased concentrations of ATP and decreased AMP-to-ATP ratio. The main effect of HFFD was an increase in AMP, resulting in increased AMP-to-ATP ratio when compared with the LFFD group.

3.6 | Alterations in amino acid and BCKA concentrations in blood plasma

In both groups of FF-treated animals, increased plasma concentrations of histidine, phenylalanine, glycine, citrulline, and ornithine and decreased concentrations of arginine and proline were found (Table 3). In the HFFD group, but not in the LFFD group, decreased concentration of all three BCAAs (valine, leucine and isoleucine), lysine, methionine, alanine, aspartate, and glutamate and increased glutamine and homocysteine were found.

Concentrations of the BCKA (α-ketoisovalerate, α-ketoisocaproate, α-keto-β-methylvalerate) were decreased in both FF-treated groups. The decrease was more pronounced in the HFFD group.

3.7 | Amino acid concentrations in the liver

In the hepatic tissue of both FF-treated groups, increased concentrations of histidine, alanine, asparagine, citrulline, ornithine, and serine and decreased aspartate were found (Table 4). Moreover, in the HFFD group, decreased leucine, methionine, proline, and valine and increased concentrations of glutamate, glutamine, and glycine were found.

TABLE 4 Effects of fenofibrate on amino acid concentration in the liver

Liver	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
EAA			
Histidine	725 ± 27	857 ± 23*	931 ± 25*
Isoleucine	161 ± 7	187 ± 7*	172 ± 6
Leucine	315 ± 15	328 ± 11	277 ± 12 [#]
Lysine	790 ± 45	1002 ± 35	994 ± 145
Methionine	75 ± 4	67 ± 2	51 ± 2*, [#]
Phenylalanine	123 ± 5	135 ± 3	120 ± 5
Threonine	1155 ± 134	1043 ± 23	1081 ± 18
Valine	337 ± 18	374 ± 12	317 ± 10 [#]
BCAA	813 ± 38	889 ± 27	766 ± 24 [#]
Σ EAA	3681 ± 201	3994 ± 49	3943 ± 177
NEAA			
Alanine	1657 ± 181	2434 ± 101*	2507 ± 168*
Arginine	65 ± 14	100 ± 15	73 ± 14
Asparagine	124 ± 8	182 ± 6*	162 ± 5*
Aspartate	1401 ± 63	1034 ± 36*	1069 ± 93*
Citrulline	81 ± 6	356 ± 80*	337 ± 68*
Glutamate	1853 ± 95	1939 ± 83	2511 ± 258*, [#]
Glutamine	4457 ± 124	5189 ± 269	6279 ± 252*, [#]
Glycine	3582 ± 91	3501 ± 106	4105 ± 190*, [#]
Ornithine	480 ± 53	1020 ± 112*	794 ± 69*
Proline	224 ± 13	247 ± 9	184 ± 11*, [#]
Serine	821 ± 60	1411 ± 142*	1434 ± 127*
Taurine	1601 ± 79	1491 ± 171	2093 ± 528
Tyrosine	161 ± 7	187 ± 7*	172 ± 6
Σ NEAA	16 390 ± 390	19 167 ± 456*	21 783 ± 755*, [#]
Σ Amino acids	20 242 ± 557	23 161 ± 477*	25 726 ± 866*, [#]

Note: The values are in nmol/g of tissue. Means ± SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids.

*Comparison vs control.

[#]HFFD vs LFFD.

3.8 | Amino acid concentrations in muscles

Decreased arginine and increased glycine were found in both muscles of both groups of FF-treated animals (Tables 5 and 6). Moreover, in EDL of HFFD-treated animals decreased lysine, methionine, proline, and the BCAA. These alterations were not observed in SOL.

4 | DISCUSSION

Our study demonstrates expected effects as well as many additional effects of FF therapy on protein, energy and amino acid metabolism in the liver and muscles. The data indicate that impaired

TABLE 5 Effect of fenofibrate on amino acid concentration in soleus muscle

SOL	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
EAA			
Histidine	348 ± 11	485 ± 24*	331 ± 24 [#]
Isoleucine	140 ± 4	135 ± 5	135 ± 5
Leucine	135 ± 2	133 ± 6	160 ± 7*, [#]
Lysine	926 ± 25	904 ± 59	767 ± 74
Methionine	58 ± 2	58 ± 2	52 ± 2
Phenylalanine	70 ± 1	80 ± 2*	94 ± 2*, [#]
Threonine	1089 ± 46	1303 ± 85	1135 ± 61
Valine	173 ± 4	177 ± 8	191 ± 8
BCAA	448 ± 9	445 ± 16	486 ± 15
Σ EAA	2939 ± 55	3275 ± 97	2865 ± 130
NEAA			
Alanine	2097 ± 73	1966 ± 59	1682 ± 80*
Arginine	380 ± 10	138 ± 12*	159 ± 9*
Asparagine	505 ± 17	501 ± 27	459 ± 25
Aspartate	3562 ± 140	3034 ± 157	1392 ± 142*, [#]
Citrulline	459 ± 12	598 ± 36*	518 ± 40
Glutamate	3686 ± 91	3267 ± 115	3567 ± 197
Glutamine	5246 ± 239	6231 ± 288*	5548 ± 195
Glycine	2782 ± 101	4788 ± 246*	7039 ± 383*, [#]
Ornithine	78 ± 3	95 ± 4*	72 ± 5 [#]
Proline	322 ± 12	306 ± 12	322 ± 11
Serine	1750 ± 82	2370 ± 203*	2168 ± 121*
Taurine	21 272 ± 529	19 045 ± 312*	18 471 ± 382*
Tyrosine	140 ± 4	135 ± 5	135 ± 5
Σ NEAA	42 069 ± 904	42 473 ± 840	41 439 ± 797
Σ Amino acids	45 009 ± 922	45 748 ± 907	44 304 ± 856

Note: The values are in nmol/g of tissue. Means ± SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids.

*Comparison vs control.

[#]HFFD vs LFFD.

muscle protein balance in HFFD-treated animals is associated with hepatomegaly, increased hepatic carnitine content, alterations in the levels of adenine nucleotides, and increased glycine and decreased BCAA, methionine, and lysine concentrations.

4.1 | Effects of FF on the liver

Hepatic enlargement in FF-treated animals is mostly due to proliferation of peroxisomes and enhanced expression of genes encoding proteins involved in lipid metabolism, resulting in decreased triglyceride concentration in the blood and the liver. The finding of the decreased hepatic triglycerides

TABLE 6 Effect of fenofibrate on amino acid concentration in extensor digitorum longus muscle

EDL	Control (n = 8)	LFFD (n = 9)	HFFD (n = 8)
EAA			
Histidine	228 ± 9	271 ± 12*	281 ± 9*
Isoleucine	161 ± 7	160 ± 5	147 ± 6
Leucine	150 ± 5	156 ± 4	137 ± 6 [#]
Lysine	615 ± 35	562 ± 40	468 ± 30*
Methionine	61 ± 2	62 ± 2	50 ± 2*, [#]
Phenylalanine	79 ± 3	90 ± 2*	85 ± 2
Threonine	1089 ± 52	1216 ± 53	1212 ± 20
Valine	214 ± 6	225 ± 6	187 ± 7*, [#]
BCAA	526 ± 16	542 ± 13	470 ± 13*, [#]
Σ EAA	2598 ± 107	2742 ± 66	2567 ± 43
NEAA			
Alanine	2172 ± 120	1782 ± 79*	1437 ± 118*
Arginine	297 ± 15	161 ± 8*	129 ± 16*
Asparagine	286 ± 16	275 ± 8	258 ± 15
Aspartate	525 ± 44	351 ± 19*	361 ± 50*
Citrulline	348 ± 15	434 ± 26	463 ± 41*
Glutamate	1884 ± 162	2054 ± 123	1972 ± 171
Glutamine	5025 ± 241	5495 ± 146	6038 ± 215*
Glycine	3435 ± 162	5953 ± 353*	6430 ± 229*
Ornithine	48 ± 2	60 ± 4*	48 ± 3 [#]
Proline	338 ± 13	321 ± 7	288 ± 7*
Serine	942 ± 42	1137 ± 50*	1105 ± 55
Taurine	16 522 ± 257	16 440 ± 365	16 757 ± 694
Tyrosine	161 ± 7	160 ± 5	147 ± 6
Σ NEAA	31 984 ± 700	34 622 ± 569*	35 433 ± 600*
Σ Amino acids	34 582 ± 771	37 364 ± 606*	38 000 ± 599*

Note: The values are in nmol/g of tissue. Means ± SE, $P < .05$. ANOVA and Bonferroni multiple comparisons.

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids.

*Comparison vs control.

[#]HFFD vs LFFD.

was an incentive to use them to treat hepatic steatosis.^{3,24,25} However, signs of hepatotoxicity in HFFD-treated animals (increased ALT and AST) and increased triglyceride content due to hepatomegaly indicate that it remains to be established whether fibrates have beneficial or adverse effect in subjects with hepatic steatosis.

The hepatic content of proteins increased proportionally with the liver weight, but not with DNA content. Therefore, the cause of hepatomegaly is hypertrophy rather than hyperplasia. The observed decrease in CHTLA in FF-treated animals and Paul and Adibi's report¹² of unchanged hepatic protein synthesis in clofibrate-fed rats indicate that decreased proteolysis plays a significant role in increased hepatic protein content.

Recent studies convincingly demonstrated that PPAR α is a key regulator of genes involved in carnitine homeostasis,²⁶ a compound essential for transport of long-chain fatty acids into the mitochondria to be oxidized. A marked increase in liver carnitine content by LFFD and HFFD (by 23- and 27-fold, respectively) is in line with earlier reports of effects of clofibrate.^{12,27} We assume that upregulation of fatty acid oxidation is the main cause of a significant increase in ATP concentration in FF-treated animals. The low hepatic AMP/ATP ratio is due to energy consumption linked to increased gluconeogenesis induced by the nocturnal starvation.²⁸

4.2 | Alterations in lysine, methionine, glycine and homocysteine concentrations are related to an increase in carnitine synthesis

A starting point in carnitine synthesis is breakdown of muscle proteins containing N6-trimethyllysine (TML) formed by post-translational methylation of lysine residues by S-adenosylmethionine (SAM) synthesized from methionine. TML released from proteins is converted to butyrobetaine and glycine, which are released to the blood. Hydroxylation of butyrobetaine to carnitine occurs only in the liver (Figure 4).

It may be suggested that enhanced catabolism of TML is the main cause of increased glycine concentration in FF-treated animals and that increased requirements of TML enhance consumption of methionine and lysine and consequently decrease their concentrations in blood plasma and muscles, as observed in animals treated by HFFD.

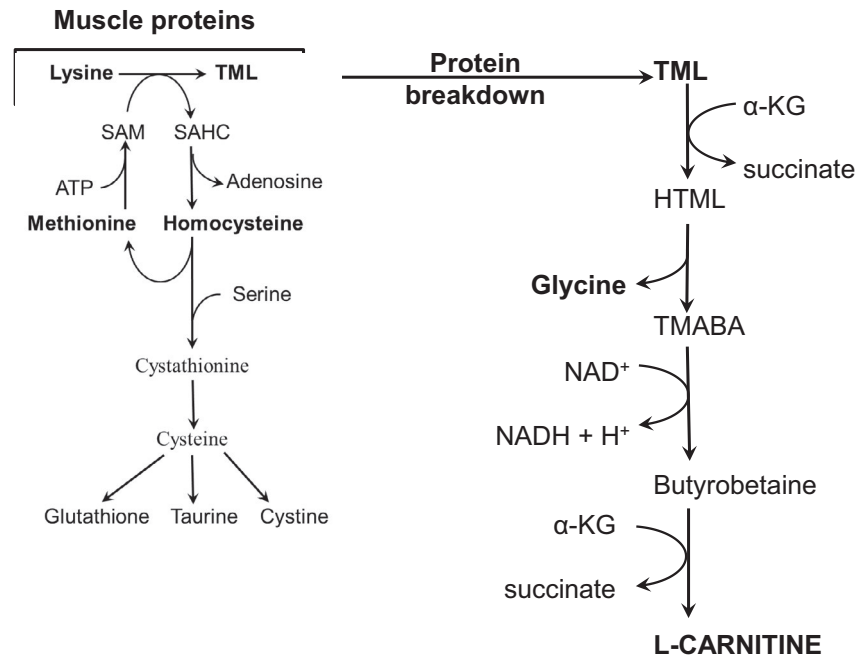
Several articles reported an FF-induced increase in blood plasma homocysteine that is recognized as an independent risk factor for atherosclerosis.^{29,30} In our study, plasma homocysteine concentration increased in HFFD-treated animals. We suppose that increased lysine methylation to TML by S-adenosylmethionine followed by production of S-adenosylhomocysteine, which is converted to homocysteine, has a role in the pathogenesis of increased homocysteine concentration in FF-treated subjects (see Figure 4).

4.3 | Alterations in BCAA and BCKA concentrations

The findings of decreased BCAA levels in blood plasma, liver, and EDL in HFFD-treated animals are in line with the observation of decreased BCAA levels in blood^{11,31} and gastrocnemius muscle of rats treated by high-dose (300 mg/kg body weight) clofibrate.¹²

The fibrates were found to inhibit the kinase responsible for phosphorylation and inactivation of branched-chain alpha-keto acid dehydrogenase, the rate-limiting enzyme in the BCAA catabolism, in the liver and muscles.^{32,33} Hence,

FIGURE 4 Role of amino acids in carnitine synthesis. Please note that lysine and methionine are the precursors for carnitine; glycine is generated in the pathway of carnitine synthesis. TML, N6-trimethyllysine; HTML, 3-hydroxy-N6-trimethyllysine; TMABA, 4-N-trimethylaminobutyraldehyde; SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine



the mechanism responsible for the decrease in BCAA is increased BCAA oxidation. A consequence and a marker of enhanced BCAA oxidation in muscles is the decrease in BCAA concentration in blood plasma, which was found in both LFFD- and HFFD-treated animals.

4.4 | Alterations in arginine

A decrease in arginine, often more than 50%, was a remarkable finding in the blood plasma and muscles of FF-treated animals. The aetiology is not clear. A possible explanation is increased use of arginine by nitric oxide synthase (NOS). The speculation is supported by increased concentrations of citrulline, which is produced in the NOS reaction (arginine + O₂ → citrulline + NO). Increased expression of the endothelial form of NOS by FF was reported in cultured rat endothelial cells and vascular endothelial cells of healthy humans after 7 days of FF therapy.^{1,2}

4.5 | Effects of LFFD and HFFD on muscles

Our findings show that weight and protein content of muscles were only slightly affected by LFFD, but markedly decreased in animals treated by HFFD. The decreased muscle mass is undoubtedly the main cause of the lower gain of body weight, which was masked by hepatomegaly in HFFD-treated animals.

The mechanism responsible for muscle wasting in FF-treated animals is obscure. Of special importance might be increased ratio of AMP to ATP observed in animals treated by HFFD, which should activate AMP-activated protein kinase

(AMPK) resulting in inhibition of the energy-consuming pathways like protein synthesis. In addition, AMPK activates autophagy,³⁴ which relates to protein degradation in lysosomes.

A role in the pathogenesis of impaired energy status and muscle protein balance during fibrate therapy might play alterations in BCAA metabolism discussed in the previous sections of the article. The studies have shown that BCAA deficiency impairs mRNA translation, and dietary inadequacies of BCAA result in impaired growth and protein wasting.^{35,36}

4.6 | Effects of FF on different muscle types

The findings of more pronounced decrease in muscle mass and muscle protein contents in EDL when compared with SOL are in a line with other reports demonstrating higher susceptibility of white, fast-twitch muscles to various catabolic stimuli than red, slow-twitch muscles.¹⁴⁻¹⁶ Furthermore, in HFFD-treated animals, a marked decrease in the BCAA, lysine and methionine concentrations was found in EDL, but not in SOL. The difference might be explained by higher protein turnover in SOL, indicated by higher CHTLA when compared with EDL, which should restore the oxidized BCAA and lysine and methionine used for carnitine synthesis.

5 | CONCLUSIONS

We conclude that FF exerts anabolic effects on hepatic protein metabolism and catabolic effects on muscles. Some

effects such as hepatic enlargement and altered concentrations of carnitine, glycine and arginine appeared both in LFFD- and HFFD-treated animals. Other effects appeared mostly in HFFD-treated animals. Of special importance are signs of hepatotoxicity, increased plasma homocysteine concentration, impaired muscle protein balance, increased AMP-to-ATP ratio, and decreased BCAA, methionine and lysine concentrations in muscles. We suppose that alterations in homocysteine, lysine, methionine and glycine are related to activated carnitine synthesis, in which lysine and methionine act as substrates and glycine is synthesized. Decreased BCAA and BCKA levels are due to increased BCAA oxidation and might be an early indication of impaired muscle protein balance. Nevertheless, recent studies have demonstrated increased BCAA levels in subjects with diabetes and suggested that increased BCAA levels play a role in development of insulin resistance and complications associated with diabetes.³⁷⁻³⁹ Therefore, the stimulatory effect of fibrates on BCAA oxidation might be of therapeutic importance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

MH outlined the experiments, performed the statistical analysis, interpreted the experimental results, and prepared the manuscript. MV was involved in the data acquisition and the data interpretation. Both authors read and approved the final manuscript.

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