Lipase maturation factor 1: Its expression in Zucker diabetic rats, and effects of metformin and fenofibrate

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Abstract

Aim. – High triglyceride (TG) levels are a risk factor for cardiovascular diseases, and TG concentrations depend on the balance between its appearance in and clearance from plasma. TG clearance is controlled mainly by lipoprotein lipase (LPL), the maturation and activity of which are dependent on lipase maturation factor 1 (LMF1) protein. The present study aimed to investigate LMF1 expression in hypertriglyceridaemia and the regulation of its expression, as little is currently known of these processes.

Methods. – We measured LMF1 expression (mRNA) in Zucker diabetic rats (ZDF) throughout the development of obesity, insulin resistance and diabetes, and compared it with that of control rats. We also determined whether fenofibrate and metformin, agents with TG-lowering activities, have an effect on LMF1 expression in ZDF rats.

Results. – At 7 weeks, the ZDF rats were obese, insulin-resistant and hypertriglyceridaemic, and their LMF1 mRNA levels – whichever tissue was investigated – were comparable to those of the control rats. Diabetic ZDF rats (14 and 21-week-old) also had high TG levels, but the presence of diabetes had no effect on LMF1 expression; mRNA levels remained comparable to those in the controls. Although fenofibrate and metformin both decreased plasma TG levels, fenofibrate had no effect on LMF1 expression, whereas metformin increased LMF1 mRNA in heart tissue (14- and 21-week-old ZDF rats; \( P < 0.01 \)), and induced a trend towards increases in adipose tissue (14-week-old ZDF rats) and muscle (14- and 21-week-old ZDF rats).

Conclusion. – LMF1 expression was not altered in this experimental animal model of obesity, insulin resistance and diabetes in the presence of raised TG levels. However, metformin increased LMF1 expression in the heart, suggesting that stimulation of LMF1 may play a part in its TG-lowering action.

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Keywords: Triglycerides; Diabetes; PPAR-α; Rat; LMF1

Résumé

Facteur 1 de maturation des lipases (LMF1) : expression chez le rat Zucker diabétique et effets du fénofibrate et de la metformine.

But. – Des concentrations élevées de triglycérides (TG) sont un facteur de risque cardiovasculaire. Ces concentrations dépendent de l’équilibre entre l’apparition et l’élimination des TG du plasma. Cette élimination est contrôlée par la lipoprotéine-lipase (LPL) dont la maturation et l’activité dépendent de la protéine Tmem112, renommée lipase maturation factor 1 (LMF1). Il n’y a pas de données sur l’expression de LMF1 dans des situations d’hypertriglycéridémie ni sur la régulation de son expression.

Méthodes. – Nous avons mesuré l’expression de LMF1 (ARNm) chez des rats Zucker diabétiques (ZDF), pendant le développement de l’obésité, de l’insulinorésistance et du diabète, comparés à des rats témoins. Nous avons déterminé si des molécules hypolipidémiantes, fénofibrate et metformine, modifiaient l’expression de LMF1 des rats ZDF.

Résultats. – À sept semaines, les rats ZDF étaient obèses, insulinorésistants et hypertriglycéridémiques ; leur concentration d’ARNm de LMF1 était dans tous les tissus comparable à celle des témoins. L’apparition du diabète (rats ZDF de 14 et 21 semaines) n’a pas modifié l’expression de LMF1 qui était toujours comparable à celle des rats témoins. La fénofibrate et la metformine ont abaissé les TG plasmatiques.
Le fénofibrate n’a pas modifié l’expression de LMF1 mais la metformine l’a augmentée dans le cœur (à 14 et 21 semaines, $P<0.01$) avec une tendance à l’augmentation dans le tissu adipeux (14 semaines) et le muscle (14 et 21 semaines).

**Conclusion.** – L’expression de LMF1 n’est pas modifiée dans le modèle d’hypertriglycéridémie du rat ZDF. La metformine stimule cette expression et cet effet pourrait contribuer à son action hypotriglycéridémiant.

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**Mots clés :** Triglycérides ; Diabète ; PPAR-α ; Rat ; LMF1

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1. Introduction

Raised triglyceride (TG) levels, or hypertriglyceridaemia, is a common lipid disorder that is often associated with obesity, insulin resistance and diabetes, and an independent risk factor for cardiovascular diseases [1]. The regulation of plasma TG metabolism is complex. Plasma TG levels result from the balance between their appearance in and clearance from the blood circulation. TG appears in plasma as TG-rich lipoproteins, as chylomicrons corresponding to intestinal secretion of absorbed dietary lipids and as very low-density lipoproteins (VLDL), corresponding to secretion of TG synthesized by the liver. Clearance of plasma TG is dependent on the action of the enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of TG present in TG-rich lipoproteins, thereby releasing fatty acids that then become available for cellular uptake by fatty acid transporters. LPL is synthesized in many different tissues, including the heart, skeletal muscle and adipose tissue. To become activated, LPL has to undergo a maturation process, involving appropriate folding and dimerization, and be transported from cells to the surface of capillary endothelium, where it is exposed to the bloodstream and can then exert its actions [2,3].

Recently, Peterfy et al. [4] identified a protein known as “Tmem112” as playing a major role in the maturation of LPL and hepatic lipase (HL), and renamed the protein “lipase maturation factor 1” (LMF1). LMF1 is expressed in most tissues, including those expressing LPL and HL. Mice deficient in LMF1 have very high TG levels due to a decrease in the activity of LPL and HL because of impaired maturation of these lipases [4]. In humans, LMF1 mutation also induces a combined lipase deficiency with hypertriglyceridaemia [4]. These data clearly show that LMF1 is involved in the regulation of both lipase activity and plasma TG concentrations.

In addition to mutation, modifications of LMF1 expression may also play a role in the control of lipase activity and plasma TG clearance. At present, little is known of the regulation of LMF1 expression and its level of expression in conditions that are classically associated with hypertriglyceridaemia, such as obesity, insulin resistance and diabetes. For this reason, we measured LMF1 expression, as reflected by mRNA levels, in an experimental model of insulin resistance, diabetes and hypertriglyceridaemia using the Zucker diabetic fatty rat (ZDF rat). We also investigated whether or not LMF1 expression could be modified by two agents known to have lipid-lowering activity: fenofibrate, a peroxisome proliferator-activated receptor-alpha (PPAR-α) agonist that increases LPL activity [5]; and metformin, an activator of 5‘-adenosine monophosphate-activated kinase (AMPk) [6].

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2. Materials and methods

2.1. Study protocols

Six-week-old ZDF rats (fa/fa) and their control littermates (C, +/+), all males, were obtained from Charles River Laboratories (L’Arbresle, France). They were housed upon arrival in an animal facility with a controlled temperature (22 ± 1 °C) and lighting (12-h light/dark cycle with lights coming on at 0700 h) and with free access to water and food throughout the study. All rats received a high-fat diet (Purina 5008 Chow; protein 26.8% of caloric value, carbohydrate 56.4% and fat 16.7%; IPS Product Supplies, London, UK) as recommended, as such a diet is necessary for the development of diabetes in male ZDF rats. Body weight was recorded twice a week, and the first metabolic investigation was performed in all rats at age 7 weeks, after 1 week of acclimatization. Thereafter, five rats from the control group and five from the ZDF group were sacrificed for blood and tissue sampling. The remaining control rats were divided in two groups (five rats each); one group was sacrificed at age 14 weeks after a second metabolic investigation, while the other group underwent metabolic investigations at age 14 and 21 weeks before being sacrificed at 21 weeks. The remaining ZDF rats were divided in three groups (10 rats each): one group received the high-fat diet only (ZDF group); the other two groups were also given either fenofibrate (ZDF + F group, 100 mg/kg/day mixed with the Chow) or metformin (ZDF + M group, 300 mg/kg/day mixed with the Chow). The addition of fenofibrate or metformin started after the first metabolic investigation (at age 7 weeks) and continued until the final sacrifice. Five rats from each of these groups were sacrificed at 14 weeks after a second metabolic investigation, and the remaining five were investigated at 14 and 21 weeks before sacrifice at 21 weeks. All experiments were conducted in agreement with the French regulations for experimentation in animals.

2.2. Metabolic investigations

Blood (250 μL, tail vein) was sampled in the fed state for measurement of blood glucose (OneTouch Ultra, LifeScan France, Issy-Les-Moulineaux, France) as well as plasma TG, non-esterified fatty acids (NEFA) and insulin.

Five rats from each group were sacrificed for tissue sampling at ages 7, 14 and 21 weeks. Food was removed at 0800 h and the rats anaesthetized at 1400 h (intraperitoneal pentobarbital 60 mg/kg) in the postabsorptive state. Blood (inferior vena cava) was collected and centrifuged, and the plasma stored at –20 °C until analysis (for NEFA and TG). Adipose tissue...
(epididymal), skeletal muscle (flexor digitorum superficialis) and the liver were quickly removed, washed with cold isotonic saline, snap-frozen in liquid nitrogen and stored at –80°C until analysis (determination of mRNA levels). The heart was washed with cold isotonic saline and the left ventricle was quickly dissected, snap-frozen in liquid nitrogen and stored at –80°C.

2.3. Analytical procedures

Plasma NEFA and TG were measured by enzymatic methods [7], and insulin by enzyme-linked immunosorbent assay (Crystal Chem, Dohners Grove, IL, USA). Total tissue RNA was purified using TRIzol reagent (Invitrogen, Cergy-Pontoise, France), with additional treatment with DNAase. Concentrations and purity were verified by measuring optical density at 230 nm, 260 nm and 280 nm, while integrity was checked by agarose gel electrophoresis. For measurements of LMF1 mRNA levels, total RNA underwent reverse transcription using Superscript II reverse transcriptase (Invitrogen) and random hexamers. Real-time polymerase chain reaction (PCR) was performed using an MyiQ thermal cycler (Bio-Rad Laboratories, Marnes-La-Coquette, France) using iQ SYBR Green Supermix (Bio-Rad). All samples were run in duplicate along with dilutions of known amounts of target sequence for quantification of initial cDNA copies. Results were expressed as the target over 18S RNA concentration ratio (ng/µg). Primer sequences for LMF1 were: forward, TTAGTCGGAGGGGACACACGTTGGA; reverse, GTCCAGGGGTTAGTGATAC; for LPL, CCTGAAGACACAGCTGAGGAC and CACCCAACTCTCATACATTC; and for 18S, GCCCTTCCACAACGTTGAGT; for HL, GCCTTCCACAACGTTGAGT; for LPL, CCTGAAGACACAGCTGAGGAC and CACCCAACTCTCATACATTC; and for 18S, GCCTTCCACAACGTTGAGT; for HSL, GCCTTCCACAACGTTGAGT. Primer sequences for LMF1 were: forward, TTAGTCGGAGGGGACACACGTTGGA; reverse, GTCCAGGGGTTAGTGATAC; for LPL, CCTGAAGACACAGCTGAGGAC and CACCCAACTCTCATACATTC; and for 18S, GCCTTCCACAACGTTGAGT; for LPL, CCTGAAGACACAGCTGAGGAC and CACCCAACTCTCATACATTC; and for 18S, GCCTTCCACAACGTTGAGT.

2.4. Statistical analysis

Results are presented as means ± SEM. Within-group comparisons of the values obtained at 7, 14 and 21 weeks in the various groups of rats (C, ZDF, ZDF + M, ZDF + F) were made by one-way analysis of variance (ANOVA), followed by the Newman–Keuls test to find differences, or by two-tailed Student’s t test for unpaired values when data were available only at 14 and 21 weeks (ZDF + M and ZDF + F groups). Between-group comparisons of the values obtained at each metabolic investigation (7, 14 and 21 weeks) were also performed by one-way ANOVA followed by the Newman–Keuls test. P < 0.05 was considered a significant difference. Statistical analyses were performed using GraphPad Prism 4.02 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Metabolic parameters

3.1.1. Body weight

At 7 weeks, the ZDF rats were obese (238 ± 5 vs 188 ± 4 g in control rats; P < 0.001), with no differences in body weight between the ZDF rats that later received either fenofibrate or metformin and those that received no treatment. Control rats gained weight between 7 and 14 weeks (to 325 ± 14 g, P < 0.01), and between 14 and 21 weeks (to 409 ± 9 g; P < 0.01). ZDF rats also increased body weight from 7 to 14 weeks (to 345 ± 5 g; P < 0.01), but less than in the controls, with a small additional increase at 21 weeks (to 361 ± 6 g). The ZDF rats’ body weights were comparable to those of control rats at 14 weeks, but lower at 21 weeks (P < 0.01), which agrees with the reference data for ZDF rats. Rats receiving fenofibrate gained less weight than the untreated ZDF group and, at 21 weeks, their weight (326 ± 6 g) was less than those of the controls and ZDF rats (P < 0.05). Metformin had no effect on body weight.

3.1.2. Glucose and insulin levels

ZDF and control rats had comparable blood glucose concentrations (7.48 ± 0.21 vs 7.35 ± 0.29 mM, respectively) at 7 weeks, although plasma insulin was higher in ZDF rats (13.9 ± 1.7 vs 4.7 ± 0.7 mU/L; P < 0.01), revealing the presence of insulin resistance. Glucose and insulin levels were unchanged in control rats at 14 and 21 weeks. Diabetes developed in all ZDF rats at around 10 weeks of age and, in all ZDF rats, glucose was > 25 mM at 14 and 21 weeks. Plasma insulin decreased in ZDF rats to values comparable to those of the controls at 14 weeks (4.2 ± 0.7 vs 6.7 ± 1.2 mU/L), and was below detection level at 21 weeks. These developments are comparable to those previously reported in ZDF rats [8]. Neither fenofibrate nor metformin modified this progression. Previous studies [9,10] have reported a reduction of hyperglycaemia with metformin in ZDF rats, but those studies used higher doses of metformin than the present study.

3.1.3. Plasma lipid concentrations

At 7 weeks, the ZDF rats had, in both fed and postabsorptive states, high TG concentrations (P < 0.001), but normal NEFA values. Plasma lipid concentrations were unchanged at 14 and 21 weeks in the control rats. NEFA concentrations (fed and postabsorptive states) were much higher in ZDF rats at 14 and 21 weeks than in either their matching 14- or 21-week-old controls, or ZDF rats before the appearance of diabetes (P < 0.01). Neither fenofibrate nor metformin modified plasma NEFA levels. Plasma TG was always higher in the ZDF than in control rats at 14 and 21 weeks. Fenofibrate and metformin decreased plasma TG concentrations in the postabsorptive state (Table 1; P < 0.05 and P < 0.01, respectively), with a more marked effect with metformin, especially at 14 weeks. However, in the fed state, metformin had no effect on plasma TG, while fenofibrate had a moderately lowering action (P < 0.05) at 14 weeks only (Table 2).

3.1.4. LMF1 mRNA levels

At 7 weeks in the presence of obesity and insulin resistance, ZDF rats had LMF1 mRNA concentrations comparable to those of 7-week-old control rats in the heart, skeletal muscle, adipose tissue and liver. In the controls, the only age-related change was a trend towards lower mRNA levels in the heart and liver that reached significance only in the liver at 14 weeks (P < 0.05) (Fig. 1). The presence of diabetes in ZDF rats (at age 14 and
21 weeks) was accompanied by no change in LMF1 mRNA levels despite a trend towards higher values in the liver. However, despite this marked trend, there was no significant difference between ZDF and control rats (P < 0.10). Fenofibrate administration to ZDF rats induced only a non-significant trend for higher values in adipose tissue. However, metformin induced a clear increase in LMF1 mRNA levels in the heart (P < 0.01 at 14 and 21 weeks). In addition, there was a trend towards higher values with metformin in skeletal muscle (14 and 21 weeks) and in adipose tissue (14 weeks), although these increases failed to reach significance due to wide interindividual variations in the metformin-treated group.

### 3.1.5. LPL and HL mRNA levels

At 7 weeks, the only difference between the ZDF and control rats was a moderate increase in LPL mRNA levels in muscle and adipose tissue (P < 0.05) (Figs. 2 and 3). Ageing induced no modifications in LPL or HL mRNA levels in control rats despite a trend towards lower LPL mRNA values in the liver. In contrast, in ZDF rats, ageing decreased LPL mRNA levels in adipose tissue (P < 0.01), with a trend towards lower values in the heart and muscle; however, no differences remained between ZDF and control rats at 14 or 21 weeks. The only significant change induced by metformin or fenofibrate administration was an increase in liver LPL mRNA at 21 weeks (P < 0.05).

### 4. Discussion

LMF1 is implicated in the maturation and activity of LPL and HL, and mutation of LMF1 in both mice and humans induces a decrease in the activity of these lipases, resulting in high plasma TG concentrations [4]. Therefore, it is possible that altering the level of expression of LMF1 could play a role in the control of plasma TG clearance and, thus, of plasma TG concentrations. We explored this possibility by measuring LMF1 mRNA levels in the heart, skeletal muscle, adipose tissue and liver of control rats and hypertriglyceridaemic ZDF rats. ZDF rats first develop obesity with insulin resistance, which is then followed by overt diabetes [8]. These metabolic abnormalities are associated with high plasma TG concentrations as a result of both increased hepatic TG synthesis and secretion, and decreased plasma TG clearance [11]. In the present experimental animal model, no decrease in LMF1 mRNA levels was observed, regardless of the tissue investigated and the exact metabolic status of the rats (insulin resistance only or overt diabetes). Although only LMF1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Evolution of triglyceride (TG) and non-esterified fatty acids (NEFA), measured in the postabsorptive state, in control and Zucker diabetic fatty (ZDF) rats, and with fenofibrate (F) and metformin (M).</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mM)</td>
<td>Control rats</td>
</tr>
<tr>
<td>7 weeks</td>
<td>0.53 ± 0.06</td>
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<tr>
<td>14 weeks</td>
<td>0.53 ± 0.07</td>
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<tr>
<td>21 weeks</td>
<td>0.59 ± 0.05</td>
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<tr>
<td>NEFA (mM)</td>
<td>Control rats</td>
</tr>
<tr>
<td>7 weeks</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>14 weeks</td>
<td>0.55 ± 0.06</td>
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<tr>
<td>21 weeks</td>
<td>0.54 ± 0.0</td>
</tr>
</tbody>
</table>

Results are presented as means ± SEM.

* P < 0.05.
** P < 0.01.
*** P < 0.001 vs corresponding control rats.
a P < 0.05.
b P < 0.01 vs corresponding ZDF rats; these parameters were measured only in the control and ZDF rats sacrificed for tissue sampling (n = 5 from each group) at age 7 weeks.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Evolution of triglyceride (TG) and non-esterified fatty acids (NEFA), measured in the Fed. State, in control and Zucker diabetic fatty (ZDF) rats, and with fenofibrate (F) and metformin (M).</th>
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</thead>
<tbody>
<tr>
<td>TG (mM)</td>
<td>Control rats</td>
</tr>
<tr>
<td>7 weeks</td>
<td>1.02 ± 0.08</td>
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<tr>
<td>14 weeks</td>
<td>1.38 ± 0.10</td>
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<tr>
<td>21 weeks</td>
<td>1.52 ± 0.12</td>
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<tr>
<td>NEFA (mM)</td>
<td>Control rats</td>
</tr>
<tr>
<td>7 weeks</td>
<td>0.57 ± 0.06</td>
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<tr>
<td>14 weeks</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>21 weeks</td>
<td>0.51 ± 0.04</td>
</tr>
</tbody>
</table>

Results are presented as means ± SEM.

** P < 0.01.
*** P < 0.001 vs the corresponding control rats.
a P < 0.05 vs the corresponding ZDF rats; for the ZDF + F and ZDF + M groups, treatment with fenofibrate and metformin, respectively, was started after the first metabolic investigation, so the 7-week values were collected before starting the treatment.
mRNA concentrations were determined, the present results do not support a role for decreased LMF1 expression in the pathogenesis of hypertriglyceridaemia – at least, not in this model.

The secondary study objective was to determine whether two agents with TG-lowering actions – fenofibrate, a PPAR-α agonist used in the treatment of hypertriglyceridaemia [7], and metformin, an activator of AMP-activated kinase [12] used
to treat type 2 diabetes [6] – could play a part by stimulating LMF1 expression. Both compounds can stimulate lipid oxidation [12,13], and decrease hepatic TG synthesis and secretion [7,12]. In addition, fenofibrate stimulates the expression of genes implicated in cellular fatty-acid uptake such as fatty-acid transporter (FAT) genes [13,14], as well as LPL expression [15] and activity [5]. Both agents decreased plasma TG levels as expected, at least in the postabsorptive state. However, there was no modification of LMF1 expression by fenofibrate. Thus, the present results do not support a role for PPAR-α in the control of LMF1 expression. Although only LPL mRNA concentrations, and not LPL activity, were measured, these data strongly suggest that the increase in LPL activity induced by fibrates [5] is not linked to altered LMF1 expression, in addition to the previously reported reduction of liver apoC-III expression [16]. Metformin, on the other hand, clearly increased LMF1 mRNA concentrations, suggesting that increased LMF1 expression might be contributory to the activity of this agent on lipid metabolism.

In conclusion, we could find no evidence of decreased LMF1 expression in an experimental model of insulin resistance and diabetes with hypertriglyceridaemia. Also, there was no evidence to support a role of PPAR-α in the control of LMF1 expression. However, metformin administration increased LMF1 mRNA concentrations, suggesting that increased LMF1 expression might be contributory to the activity of this agent on lipid metabolism.