Effect of insulin treatment on plasma oxidized LDL/LDL-cholesterol ratio in type 2 diabetic patients

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SUMMARY
Objective: In type 2 diabetes mellitus, oxidized LDL/LDL-Cholesterol ratio, an accurate estimation of in vivo LDL oxidation, has been reported elevated and associated with macrovascular disease. Because insulin therapy induces significant modification of lipid metabolism, in type 2 diabetes, we evaluated the effect of insulin treatment on oxidized LDL/LDL-C ratio in type 2 diabetic patients and analyzed the results in comparison with the modifications induced by insulin on glycaemia, plasma lipids and LDL receptors.

Research design and Methods: Plasma oxidized LDL concentrations were measured by sandwich ELISA in 21 type 2 diabetic patients before and 3 months after the introduction of insulin therapy, and in 27 age-matched controls.

Results: Type 2 diabetic patients had, compared to controls, significantly increased oxidized LDL/LDL-C ratio (P<0.0001). Three months after insulin treatment, oxidized LDL/LDL-C ratio was significantly reduced (21.1±4.7 vs. 24.0±5.8 U/mmol, P<0.01). This reduction was strongly associated, in multivariate analysis, with reduction of LDL(TG/cholesterol ratio) (P=0.008), and to a lesser extent with the decrease of LDL fructosamine (P=0.034), but not with the increase of the number of LDL receptors.

Conclusions: In the present study we demonstrate for the first time a lowering effect of insulin therapy on oxidized LDL/LDL-C ratio in type 2 diabetic patients. This decrease is mainly associated with the reduction of LDL TG-enrichment, and to a lesser extent with the decrease of LDL glycation, but not with the insulin-induced increase in number of LDL receptors.

Key-words: Lipoproteins · Oxidized LDL · Type 2 diabetes · Insulin · Atherosclerosis.
Introduction

Macrovascular disease is the leading cause of increased morbidity and mortality in patients with type 2 diabetes [1,2]. Lipid abnormalities play an important role in the development of atherosclerosis in type 2 diabetic patients [3]. The dyslipidemia observed in type 2 diabetes includes both quantitative and qualitative lipid abnormalities [4-6]. Quantitative lipid abnormalities are represented by hypertriglyceridemia and decreased plasma HDL-C levels [6]. Qualitative abnormalities include the presence of small dense LDL particles [6,7], increased triglyceride content of LDL and HDL [6,8], glycation of apolipoproteins [6,8-10], and increased susceptibility of LDL to oxidation [5,6,11]. These lipid qualitative abnormalities are likely to promote atherosclerosis. Among these, the oxidative process, particularly the oxidative conversion of native LDL to oxidized LDL, is now considered to be an essential step in the atherogenic process [12,13]. Increased circulating levels of oxidized LDL have been found in type 2 diabetes [14,15]. Oxidized LDL/LDL-C ratio, which is an accurate estimation of lipid oxidation for each LDL particle, independently of plasma LDL-C level, has been found associated with macrovascular disease in type 2 diabetes [16].

Insulin plays a central role in the regulation of lipid metabolism [17]. Indeed, insulin is a potent activator of lipoprotein lipase, inhibits VLDL production by the liver in vitro [18,19] and in vivo [20,21] and promotes the clearance of LDL [22-25]. In type 2 diabetic patients, treatment with insulin has been shown to induce significant modifications of lipid metabolism [26,27]. A decrease of serum total and VLDL triglyceride levels has been shown in type 2 diabetic patients after insulin therapy [28]. Our group has previously shown that insulin therapy, in type 2 diabetes, significantly reduces the triglyceride-rich lipoprotein pool by increasing both VLDL and IDL catabolism [26]. Insulin therapy has also been shown to significantly improve LDL metabolism in type 2 diabetic patients [26,29]. Indeed, LDL catabolism which is significantly reduced in type 2 diabetic patients on oral agents is totally normalized by insulin therapy [26,30]. This beneficial effect of insulin on LDL catabolism could be partly related to the significant increase in LDL-receptors on cell surface induced by insulin treatment in type 2 diabetic subjects [29]. Thus, insulin treatment induces important modification of lipid metabolism in type 2 diabetes. So far, the effect of insulin therapy on oxidized LDL/LDL-C ratio has not yet been studied.

Since LDL oxidation is a potential important factor in the development of atherosclerosis in type 2 diabetes and since insulin therapy induces significant modification of lipid metabolism in type 2 diabetic patients, we performed a prospective study whose aim was to analyze the effect of insulin treatment on plasma oxidized LDL/LDL-C ratio, in type 2 diabetes, in comparison with the modifications induced by insulin on glycaemia, plasma lipids and LDL-receptors.

Research design and methods

Patients

Twenty one type 2 diabetic patients and 27 healthy non-diabetic normolipidemic control subjects were studied.

Type 2 diabetic patients were studied before and 3 months after insulin therapy. Type 2 diabetic patients were, at entry, all treated with oral antidiabetic drugs (both glibenclamide 15 mg/day and metformin 2.550 mg/day). They were not taking hypolipidemic drugs or medication known to modify lipid metabolism.

All control subjects were in good health, with normal tolerance and normal plasma lipid levels. They were not taking any medication. In both groups, subjects with liver disease, renal failure or thyroid disease were excluded.

The 21 diabetic patients were treated by insulin because of failure of oral antidiabetic therapy. The insulin regimen consisted of 2 daily injections of intermediate-acting human insulin at a dose of 0.30-1.5 units/kg/day. The patients performed capillary glucose monitoring three times per day. They were educated in order to adapt insulin doses according to capillary blood glucose levels. The goal was to obtain a fasting blood glucose level between 4.5 and 8.25 mmol/l. The patients were asked to increase their insulin dose by 2 units when fasting glycaemia was >8.25 mmol/l and to reduce it by 2 units when fasting glycaemia was <4.5 mmol/l.

A metabolic assessment was performed in the 21 type 2 diabetic patients and the 27 control subjects including fasting blood glucose, plasma lipids, oxidized LDL/LDL-C ratio, LDL size and LDL composition. A second metabolic assessment was performed 3 months after initiation of insulin therapy (the blood sampling was done before insulin administration). Moreover, a measurement of LDL-receptors on cell surface was made in these 21 patients before and 3 months after introduction of insulin treatment.

The protocol was approved by the Dijon University Hospital ethics committee and written informed consent was obtained before the study was started.

Plasma-Oxidized LDL Concentration

Oxidized LDL was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) in which two monoclonal antibodies were directed against separate antigenic determinants on the apolipoprotein B molecule. The ELISA assay used is commercially available and was used according to the manufacturer’s instructions. The interassay coefficient of variance (CV) of the assay was 8%. In order to have an accurate estimation of oxidation in each LDL particle, results were expressed as oxidized LDL/LDL-C ratio, as previously reported [16,31].

Isolation of lipoprotein subfractions

Serum lipoproteins were isolated by sequential ultracentrifugation in a 50.4 rotor (on an L7 ultracentrifuge Beckman, Palo Alto, CA) using the following densities (d): VLDL...
Table I
Clinical and plasma biological characteristics of type 2 diabetic patients before insulin therapy and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Type 2 diabetic patients before insulin therapy</th>
<th>Control subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>70.0±11.3</td>
<td>50.1±14.12</td>
<td>0.002</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>7/14</td>
<td>9/18</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6±3.5</td>
<td>24.7±4.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>12.6±3.3</td>
<td>5.0±0.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.4±1.4</td>
<td>0.8±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6±1.5</td>
<td>5.2±0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.2±0.3</td>
<td>1.5±0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.4±1.1</td>
<td>3.3±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>LDL(TG/cholesterol ratio)</td>
<td>0.2±0.1</td>
<td>0.1±0.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>27.7±1.3</td>
<td>30.4±2.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Oxidized LDL/LDL-C ratio (U/mmol)</td>
<td>23.5±6.1</td>
<td>15.6±2.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are means ±SD; NS non significant.

d<1.006 g/ml, IDL 1.006<d<1.019 g/ml, LDL 1.019<d<1.063 g/ml, and HDL 1.063<d<1.210 g/ml. Densities were adjusted by adding potassium bromide.

Biochemical analysis

Plasma glucose concentrations were measured by an enzymatic method (glucose oxidase) on a vitros 750 analyzer (Ortho Clinical Diagnostics, Rochester, NY). Fructosamine, total and HDL-C, triglyceride, and apoB concentrations were measured in a Cobas Integra analyzer (Roche, Basel, Switzerland) with dedicated reagents. LDL-C concentrations were calculated using the Friedewald formula [34].

Determination of LDL size

The mean apparent diameter of plasma LDL particles was determined by non denaturing electrophoresis in 20-160 g/l polyacrylamide gradient gels. At the end of the electrophoresis, the gels were fixed, stained with coomassie brilliant blue G, and destained. The distribution profile of LDL was finally obtained by densitometric scanning of the gels on a BioRad GS-670 imaging densitometer. The apparent diameter of the predominant LDL subfraction was determined by comparison with ferritin (diameter, 12.20 nm; Pharmacia), thyroglobulin (diameter, 17.00 nm; Pharmacia), and carboxylated latex beads (diameter, 38.00 nm; Duke scientific) that were subjected to electrophoresis with the LDL samples [35].

LDL-receptor measurement

LDL-receptor expression was quantified by a flow cytometry method using monoclonal anti-LDL receptor antibodies on peripheral monocytes isolated from blood samples immediately after blood drawing, as previously described [29]. The inter and intra CV of the assay were 5%.

Statistical analysis

Results are expressed as means ± SD. Statistical calculations were performed using the SPSS software package. Comparison of means between controls and type 2 diabetic patients was performed using Student’s t test. Comparison of means between diabetic patients and controls, after adjustment for age, was performed by analysis of covariance. Comparison of percentages between diabetic patients and controls was performed by Chi-square test. Comparison of means before and after insulin therapy, in type 2 diabetic patients, was performed using the paired Student’s t test. Correlation coefficients were calculated by the Spearman test. Multivariate analysis was made by stepwise multiple linear regression. P values <0.05 were considered statistically significant.

Experimental results

Comparison between type 2 diabetic patients, before insulin therapy, and control subjects

Clinical and biological characteristics (table I)

Clinical and glucose metabolism characteristics are represented in table I. Type 2 diabetic patients were significantly overweight compared to control subjects (BMI 28.6±3.5 vs. 24.7±4.1 kg/m², P=0.001). Type 2 diabetic patients presented a 2.5-fold increase in fasting plasma TG compared with control subjects (2.4±1.4 vs. 0.8±0.3 mmol/l,
P<0.0001). Total cholesterol and LDL-C concentrations were comparable between diabetic patients and control subjects. Mean HDL-C concentrations was lower in type 2 diabetic patients compared with control subjects (1.2±0.3 vs. 1.5±0.4 mmol/l, P=0.001) (table I).

**LDL particle composition and size (table I)**

LDL(TG/cholesterol ratio) was significantly increased in type 2 diabetic patients compared with control subjects (0.2±0.1 vs. 0.1±0.0 mmol/l, P<0.0001). LDL size was significantly reduced in type 2 diabetic patients (27.7±1.3 vs. 30.4±2.0 nm, P<0.0001) (table I).

**Oxidized LDL/LDL-C ratio (table I)**

Oxidized LDL/LDL-C ratio, which represents an accurate estimation of lipid oxidation for each LDL particle, independently of plasma LDL-C level, was significantly increased in type 2 diabetic patients compared with controls (23.5±6.1 vs. 15.6±2.8 U/mmol, P<0.0001). These differences remained highly significant after adjustment for age (P<0.0001) (table I).

**Effect of insulin therapy in type 2 diabetic patients**

**Clinical and biological characteristics (table II)**

After 3 months of insulin therapy, type 2 diabetic patients significantly improved their glycemic control, as assessed by the reduction of both mean fasting blood glucose concentration (10.4±3.2 vs. 12.6±3.3 mmol/l, P<0.05), and HbA1c (8.7±1.5 vs. 9.9±2.0 %, P<0.05) (table I). After 3 months of insulin therapy, plasma TG fell by 25% (1.8±0.8 vs. 2.4±1.4 mmol/l, P<0.05). Total cholesterol and LDL-C concentrations were not significantly modified by insulin therapy. HDL-C concentration increased significantly on insulin therapy (1.4±0.4 vs. 1.2±0.3 mmol/l, P<0.001) (table II).

**LDL particle composition and size (table II)**

LDL(TG/cholesterol ratio) was significantly reduced after insulin therapy (0.13±0.10 vs. 0.22±0.10, P<0.05). LDL fructosamine/apoB ratio was also significantly reduced after insulin therapy (8.5±3.0 vs. 10.5±3.8, P<0.001). LDL size increased significantly after insulin therapy (28.2±1.4 vs 27.8±1.4 nm, P<0.01) (table II).

**Oxidized LDL/LDL-C ratio (table II and figure 1)**

Oxidized LDL/LDL-C ratio decreased significantly after insulin therapy (21.1±4.7 vs. 24.0±5.8 U/mmol, P<0.01). Oxidized LDL/LDL-C ratio, in type 2 diabetic patients after insulin treatment, remained higher than in controls.

**Monocyte LDL-receptors (table II)**

After 3 months of insulin therapy, LDL-receptor expression per cell (monocyte) increased by 57% (10,096±5,657 vs. 6,439±2,310, P<0.01).

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**Table II**

Clinical and plasma biological characteristics of type 2 diabetic patients before and 3 months after insulin therapy.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients before insulin therapy</th>
<th>Diabetic patients after 3 month insulin therapy</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>70.0±11.3</td>
<td>70.0±11.3</td>
<td>-</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>6/15</td>
<td>6/15</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.4±3.5</td>
<td>29.2±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>12.6±3.3</td>
<td>10.4±3.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.9±2.0</td>
<td>8.7±1.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.4±1.4</td>
<td>1.8±0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6±1.5</td>
<td>5.8±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.2±0.3</td>
<td>1.4±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.4±1.1</td>
<td>3.7±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>LDL(TG/cholesterol ratio)</td>
<td>0.22±0.10</td>
<td>0.13±0.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL (fructosamine/apoB ratio)</td>
<td>10.5±3.8</td>
<td>8.5±3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL size (nm)</td>
<td>27.8±1.4</td>
<td>28.2±1.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oxidized LDL/LDL-C ratio (U/mmol)</td>
<td>24.0±5.8</td>
<td>21.1±4.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL receptors (per monocyte)</td>
<td>6,439±2,310</td>
<td>10,096±5,657</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are means ±SD; NS non significant.
Insulin treatment and oxidized LDL-cholesterol

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Univariate and Multivariate analyses

A strong correlation between the reduction of oxidized LDL/LDL-C ratio after insulin therapy and the reduction of LDL (TG/cholesterol ratio) was noted ($r=0.69; P<0.001$). We didn’t find any correlation between the reduction of oxidized LDL/LDL-C ratio, on the one hand, and the modification of LDL size, HbA$\text{$_1C$}$, plasma HDL-C, LDL receptor and LDL fructosamine, on the other hand.

In the multivariate analysis, the decrease of oxidized LDL/LDL-C ratio after 3 months insulin therapy was independently associated with both the reduction of LDL (TG/cholesterol ratio) ($P=0.008$) and the decrease of LDL fructosamine ($P=0.034$), but not with LDL-receptor, plasma HDL-C, and fasting blood glucose modifications after insulin therapy. The two factors (LDL fructosamine, and LDL (TG/cholesterol ratio) explained 50% of the oxidized LDL/LDL-C ratio decrease. (table III).

Discussion

In the present study, we confirm, as previously reported, that oxidized LDL/LDL-C ratio is significantly increased in type 2 diabetic patients and we demonstrate for the first time that insulin treatment significantly reduces plasma oxidized LDL/LDL-C ratio in type 2 diabetic patients.

The significant increase of oxidized LDL level could participate to the development of atherosclerosis in diabetic patients. Indeed, many studies have shown the deleterious effects of oxidized LDL on the vascular wall [12] and pointed out that subjects with elevated blood levels of oxidized LDL may be at risk for acute coronary events [36]. Moreover, recently it has been reported that subjects with high oxidized LDL showed a greater disposition to myocardial infarction [33]. In type 2 diabetes, previous studies have confirmed a relation between LDL susceptibility to oxidation and atherosclerosis [13,39]. More recently with the development of immunoassays which measure directly oxidized LDL particles, increased circulating levels of oxidized LDL have been found in type 2 diabetes [14,15]. Furthermore, oxidized LDL/LDL-C ratio, an accurate estimation of lipid oxidation in vivo in each LDL particle, has been found associated with cardiovascular disease in type 2 diabetes [16]. Oxidized LDL/LDL-C ratio has been shown to be an significant and independent marker of cardiovascular risk [31].

We show, in our study, that insulin therapy, in type 2 diabetic patients, induces a significant reduction of oxidized LDL/LDL-C ratio, indicating a decrease in oxidation in each LDL particle. This reinforces the previously described positive effects of insulin on lipid abnormalities in type 2 diabetes such as reduction of hypertriglyceridemia and LDL-TG.

Figure 1
Oxidized LDL/LDL-C ratio in 21 type 2 diabetic patients before and 3 months after insulin treatment. Values represent the mean number (±SD). Mean significantly decreases in type 2 diabetic patients after insulin treatment ($P<0.01$, paired t test).

Table III
Multivariate analysis. Multiple linear regression with oxidized LDL/LDL cholesterol ratio modification after insulin therapy in type 2 diabetic patients as dependent variable.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SD</th>
<th>t</th>
<th>P</th>
<th>$R^2$ of the model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significant and independent variables</td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (TG/chol. ratio) variation</td>
<td>46.043</td>
<td>14.803</td>
<td>3.10008</td>
<td></td>
</tr>
<tr>
<td>LDL fructosamine variation</td>
<td>1.099</td>
<td>0.467</td>
<td>2.30034</td>
<td></td>
</tr>
</tbody>
</table>

Variables introduced into the model: LDL (TG/chol. ratio) variation, LDL fructosamine variation, HbA$\text{$_1C$}$ variation, % of LDL-receptors variation and plasma HDL-C variation. SD: Standard Deviation.
enrichment [27-29]. Our prospective study of the effect of insulin on LDL oxidation in patients with Type 2 diabetes confirms the potential antioxidant properties of insulin which might be suspected from the study by Kondo et al. who showed lower plasma levels of malondialdehyde-modified LDL/ apoB ratio in diabetic patients treated with insulin [40].

As previously reported, insulin treatment significantly increases LDL-receptor number in type 2 diabetic patients [29]. However, in our study, the decrease of oxidized LDL/LDL-C ratio induced by insulin is mainly explained by the reduction of TG-content in LDL particles and to a lesser extent by the decrease of LDL glycation, but not by the increase of the number of LDL-receptors. We have previously shown by in vivo stable isotope kinetic studies [26] that the increase of LDL-receptors induced by insulin treatment in type 2 diabetic patients have positive effects such as an increase of LDL catabolism. However, such an increase of the number of LDL-receptors, although significantly reducing LDL plasma residence time is not responsible for the decrease in plasma oxidized LDL/LDL-C ratio.

The decrease of TG-content in LDLs plays an important role in the reduction of oxidized LDL/LDL-C ratio following insulin treatment in type 2 diabetic patients. Several studies have shown, in type 2 diabetic patients, positive action of insulin on TG-rich lipoprotein metabolism, including a reduction of TG-rich lipoprotein pool and LDL TG-content [21,26]. It has been shown that elevated TG-content within LDL particles increases LDL susceptibility to oxidation [41,42]. Reduction of LDL TG-content on insulin therapy reduces LDL susceptibility to oxidation and thus decreases oxidized LDL/LDL-C ratio. Furthermore, we show that the significant reduction of LDL glycation with insulin treatment is associated with the reduction of oxidized LDL/LDL-Cholesterol ratio. Since glycated LDL particles are known to be more susceptible to oxidation [39], we may think that the decrease of LDL glycation induced by insulin treatment is likely to play a role in the reduction of their oxidation.

Other mechanisms could also be involved in the reduction of oxidized LDL/LDL-Cholesterol ratio by insulin therapy such as anti-inflammatory properties of insulin. Indeed, it has been shown that insulin has anti-inflammatory properties [43-45]. In patients with type 2 diabetes, treatment with insulin, but not improved glycaemic control per se, has been shown to reduce circulating C-Reactive Protein, which is in favor of a direct effect of insulin on the hepatic acute phase response. Moreover, it has been shown that insulin has a potent acute anti-inflammatory effect including decrease in reactive oxygen species (ROS) generation, a reduction in intranuclear nuclear factor κB (NFκB) and other inflammatory mediators including soluble intercellular adhesion molecule-1 (sICAM-1) [44]. In addition insulin has been shown to inhibit Tumor Necrosis Factor (TNF), in vitro [46]. Thus, we cannot exclude that insulin itself may directly play a role in the reduction of LDL oxidation.

Conclusions

In conclusion, we demonstrate for the first time that insulin therapy significantly reduces plasma oxidized LDL/LDL-C ratio in type 2 diabetic patients. This decrease is mainly associated with a reduction of LDL-triglyceride levels and to a lesser extent with the decrease of LDL glycation, but not with the insulin-induced increase of number of LDL-receptors. This study reinforces the positive effect of insulin therapy in type 2 diabetic patients not only on glucose metabolism but also on lipids.

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