ORIGINAL ARTICLE

Evaluation of paraoxonase 1 arylesterase activity and lipid peroxide levels in patients with type 1 diabetes

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KEY WORDS

late diabetic complications, lipid peroxide, paraoxonase 1, oxidative stress, type 1 diabetes

ABSTRACT

INTRODUCTION Poor metabolic control of type 1 diabetes is one of the most important factors accelerating the development of late diabetic complications. Several other factors that might contribute to this process are currently being investigated. Low paraoxonase 1 (PON1) activity and high lipid peroxide (LPO) levels contribute to endothelial damage, but it remains unclear whether they are critical for the development of late diabetic complications.

OBJECTIVES The aim of the study was to evaluate PON1 arylesterase activity and LPO levels in patients with type 1 diabetes and to investigate whether these parameters are associated with metabolic control and late complications. Moreover, we aimed to establish whether PON1 activity and LPO levels differ between women and men with type 1 diabetes.

PATIENTS AND METHODS The study involved 80 patients with type 1 diabetes and 24 healthy subjects. PON1 activity was measured by a spectrophotometric method. LPO levels were measured by a commercial assay kit.

RESULTS Diabetic patients had lower PON1 activity and higher LPO levels than healthy people. We observed a negative correlation between PON1 activity and LPO levels in diabetic patients. There was no association between PON1 activity or LPO levels and metabolic parameters or late diabetic complications. There was a positive correlation between LPO levels and the body mass index (BMI) in women with type 1 diabetes.

CONCLUSIONS Our study showed that low PON1 activity and high LPO levels are not the most critical factors involved in late diabetic complications in type 1 diabetes. Increased LPO levels in women with type 1 diabetes may result from enhanced lipogenesis in this subgroup compared with diabetic men.

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INTRODUCTION Type 1 diabetes is associated with the development of late diabetic complications.[1,2] High long-term levels of hemoglobin A_{1c} (HbA_{1c}) and diabetes duration are the most important (but not the only) independent risk factors for late diabetic complications.[3,4] This explains the search for other factors that may affect the progression of this harmful process.

Recently, it has been observed that lipid disorders can play a significant role in the development of late diabetic complications.[5] Clinical results show that patients with type 1 diabetes have impaired lipid profile.[6,7] However, contrary to type 2, in type 1 diabetes the changes are subtle and more often associated with qualitative rather than quantitative lipid abnormality.[8] It has been established that high-density lipoprotein (HDL) shows decreased capacity to protect against lipid oxidation in patients with type 1 diabetes compared with healthy people.[9] It is partially related to the low activity of HDL-associated antioxidant enzyme – paraoxonase 1 (PON1).[10] PON1 is

a calcium-dependent esterase exclusively bound to the apolipoprotein A1-containing HDL fraction in plasma.[11] This enzyme has a vasculoprotective function by hydrolyzing preformed LPO and the capacity to inhibit the oxidation of lipoprotein process.[12] The best explained mechanism through which PON1 shows protective role is hydrolysis of homocysteine-thiolactone (Hcy-thiolactone). This molecule, by binding with protein lysine residues, generates N-Hcy-protein with autoimmunogenic and prothrombotic properties. Therefore, hydrolysis of Hcy-thiolactone mediated by PON1 decreases harmful modification of endothelial proteins and protects against atherosclerosis.[13]

Low PON1 activity increases lipid oxidation in patients with type 1 diabetes,[14,15] the process that is postulated to play a central role in endothelial cell damage.[16] Therefore, low activity of PON1 could contribute to vascular dysfunction and late diabetic complications in patients with type 1 diabetes.

The aim of the study was to evaluate PON1 activity and LPO levels in patients with type 1 diabetes and to investigate whether these parameters are associated with metabolic control and late diabetic complications. Moreover, we aimed to establish whether PON1 activity and LPO levels differ between women and men with type 1 diabetes.

PATIENTS AND METHODS

Study groups The study group consisted of 80 patients with type 1 diabetes, aged 34.4 ±6.3 years. Diabetes was diagnosed on the basis of typical symptoms, blood glucose levels higher than 11.1 mmol/l, and C-peptide levels lower than 0.5 µg/l.[17] All patients were treated with intensive insulin therapy (IIT) from the onset of the disease. For IIT, fast- and long-acting insulin has been used. Patients presented for follow-up every year at the Department of Internal Medicine and Diabetology at the Poznan University of Medical Sciences, Poznań, Poland.[18]

The measurement of PON1 activity and LPO levels was performed after 10.0 ±2.0 years since diagnosis. Lipid profile parameters were within the reference range. Glycemic control in most cases was not satisfactory (HbA_{1c} 8.1 ±1.6%) to maintain good metabolic control. The body mass index (BMI) of the patients was 23.5 ±3.5 kg/m^2.

The study group was divided according to sex (28 women, 52 men) and according to the absence (n = 44) or presence (n = 36) of late diabetic complications including retinopathy (n = 25), nephropathy (n = 27), and neuropathy (n = 11). In 21 patients, more than one type of late diabetic complications have been diagnosed.

Diabetic retinopathy was established by direct ophthalmoscopy through dilated pupils followed in all patients by fundus photography.

Diabetic nephropathy was detected at the stage of albuminuria (urinary albumin excretion rate between 30 and 300 mg/24 h in 2 samples collected over a 3-month period after exclusion of secondary causes of microproteinuria). Diabetic kidney disease was defined as the presence of albuminuria associated with diabetes of over 10-year duration, or with diagnosed diabetic retinopathy.

Diabetic neuropathy was diagnosed in patients with 2 or more of the following components: the presence of symptoms of neuropathy, the absence of ankle tendon reflexes, and/or abnormal scores for pressure and/or vibration perception.[18]

The control subjects were 24 blood donors (12 women, 12 men), aged 31.1 ±10.8 years, who underwent a medical check-up before having blood taken in the fasting state. They were characterized by the reference levels of lipid profile and glucose levels, and were not overweight (BMI 21.5 ±1.0 kg/m^2).

The study protocol was approved by the Ethics Committee of the Poznan University of Medical Sciences (No 871/08) and written informed consent was obtained from all participants.

Blood samples were collected in tubes without anticoagulant. The samples were allowed to clot at room temperature and then centrifuged at 2000 g for 15 minutes to obtain serum. All the serum samples were stored at –80° C until analysis.

Metabolic parameters The levels of triglycerides (TG), total cholesterol (TC), HDL cholesterol (HDL-C), and glucose were determined using the commercially available assay kits (Roche, CH) with an analyzer (Cobas6000, Roche, CH). LDL cholesterol (LDL-C) was obtained using the following formula: LDL-C = TC – HDL-C – TG/5. HbA_{1c} was measured using high-performance liquid chromatography with the Variant Hemoglobin A1c Program (Bio-Rad Laboratories, Hercules, CA, United States).[19]

Determination of lipid peroxide concentration LPO was quantified with an LPO assay kit (Cayman Chemical, Ann Arbor, United States), which measures LPO directly utilizing the redox reactions with ferrous ions.[20] Briefly, 500 µl of serum was added to 2 ml of chloroform and 1 ml of methanol. The solution was mixed thoroughly and then centrifuged at 1500 × g for 5 minutes in 4°C to extract LPO to the chloroform layer. Then, 500 µl of extract was mixed with 450 µl of chloroform-methanol solvent and 50 µl of freshly prepared chromogen (4.5 mM ferrous sulfate in 0.2 M hydrochloric acid) in a glass tube. Absorbance was measured at 500 nm after 5-minute incubation. LPO levels in the samples were calculated from a standard curve of LPO. Each serum was analyzed in duplicate, and LPO levels were expressed as µmol/ml.

Determination of paraoxonase 1 activity PON1 arylesterase activity was measured by a spectrophotometric method.[21] Briefly, the assay was run in a cuvette in 20 mM Tris-HCl buffer containing

TABLE 1 Characteristics of patients with type 1 diabetes and the control group

	Patients (n = 80)	Control group (n = 24)	*P*
sex, (female/male)	28/52	12/12	0.001
age, y	34.4 ±6.3	31.1 ±10.8	NS
diabetes duration, y	10.0 ±2.0	–	–
functional insulin therapy, %	100	–	–
insulin dose, units/kg body weight/day	0.63 ±0.19	–	–
ACEI, %	31	–	–
BMI, kg/m^2	23.5 ±3.5	21.5 ±1.0	NS
FPG, mmol/l	9.3 ±2.8	4.5 ±0.5	0.0001
PPG, mmol/l	8.9 ±2.1	–	–
HbA_{1c}, %	8.1 ±1.6	–	–
TG, mmol/l	0.9 (0.6–1.3)	1.0 (0.9–1.2)	NS
TC, mmol/l	4.5 (4.1–5.3)	4.0 (3.6–4.6)	0.0005
LDL-C, mmol/l	2.8 (2.3–3.4)	2.2 (2.0–2.6)	0.001
HDL-C, mmol/l	1.7 (1.4–2.0)	1.3 (1.2–1.4)	0.0001
late diabetic complications, n (%)	36 (45)	–	–
retinopathy	25 (31.25)	–	–
nephropathy	27 (33.75)	–	–
neuropathy	11 (13.75)	–	–

Data are given as mean ± SD or median (interquartile range).

Abbreviations: ACEI – angiotensin-converting enzyme inhibitors, BMI – body mass index, FPG – fasting plasma glucose, HbA_{1c} – glycated hemoglobin A_{1c}, HDL-C – high-density lipoprotein cholesterol, LDL-C – low-density lipoprotein cholesterol, NS – nonsignificant, PPG – postprandial plasma glucose, SD – standard deviation, TG – triglycerides, TC – total cholesterol

1.0 mM CaCl2 and 1.0 mM phenyl acetate. The reaction was initiated by the addition of the enzyme (5 µl of serum), and the increase in absorbance at 270 nm was recorded. To correct the results of absorbation caused by spontaneous hydrolysis of phenyl acetate, blank sample without enzyme was used. Arylesterase activity was calculated using the molar extinction coefficient (ε = 1310 M-1 cm-1) of phenol produced. A unit of arylesterase activity was defined as 1 µmol of phenyl acetate hydrolyzed per minute under the above assay conditions.

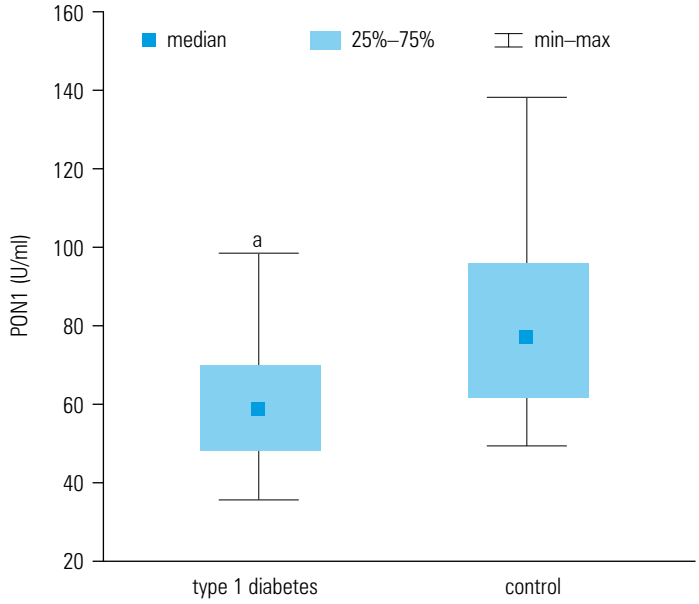

FIGURE 1 Paraoxonase 1 (PON1) activity in patients with type 1 diabetes and in controls; the Mann–Whitney test; **a** $P = 0.001$

Statistical analysis Data are expressed as means ± standard deviation or median with the lower–upper quartile, depending on the type of distribution. For comparing clinical parameters with normal distribution, the *t* test was used. For parameters without normal distribution the Mann–Whitney test was applied. The Spearman correlation coefficient was used to test whether PON1 activity and LPO levels are associated with metabolic control and late diabetic complications. The level of $P < 0.05$ was considered statistically significant.

RESULTS Clinical and metabolic characteristics of the patients are presented in TABLE 1. Patients with type 1 diabetes had higher fasting plasma glucose, TC, and LDL-C than the control group (9.3 ±2.8 vs. 4.5 ±0.5 mmol/l, $P = 0.0001$; 4.5 [4.1–5.3] vs. 4.0 [3.6–4.6] mmol/l, $P = 0.0005$; 2.8 [2.3–3.4] vs. 2.2 [2.0–2.6] mmol/l, $P = 0.001$, respectively). HDL-C levels were also higher in diabetic patients than in the control group (1.7 [1.4–2.0] vs. 1.3 [1.2 – 1.4], $P = 0.0001$) (TABLE 1).

The arylesterse activity of PON1 was lower in diabetic patients than in the control group (61.4 [48.1–69.9] vs. 77.6 [61.4–95.8] U/ml, $P = 0.0001$] (FIGURE 1).

Moreover, patients with diabetes had higher LPO levels than control subjects (4.1 [1.6–6.0] vs. 1.0 [0.3–2.2] µmol/l, $P = 0.0001$] (FIGURE 2).

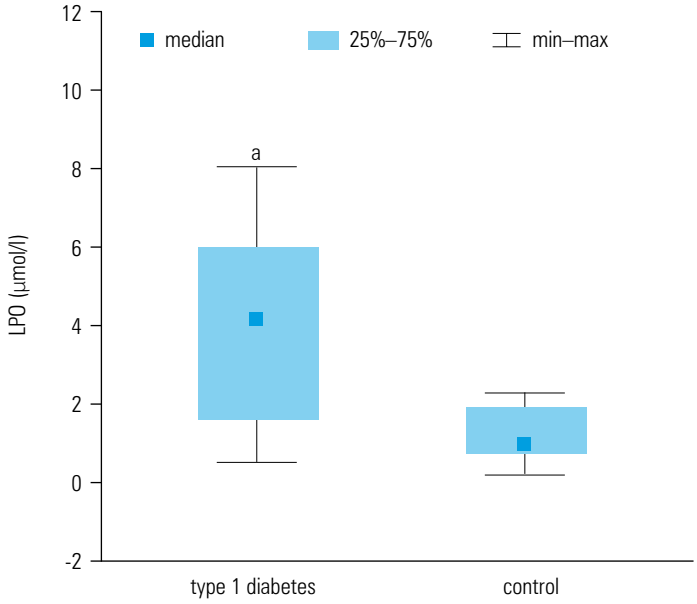

FIGURE 2 Lipid peroxide levels (LPO) in patients with type 1 diabetes and in controls; the Mann–Whitney test; a $P = 0.0001$

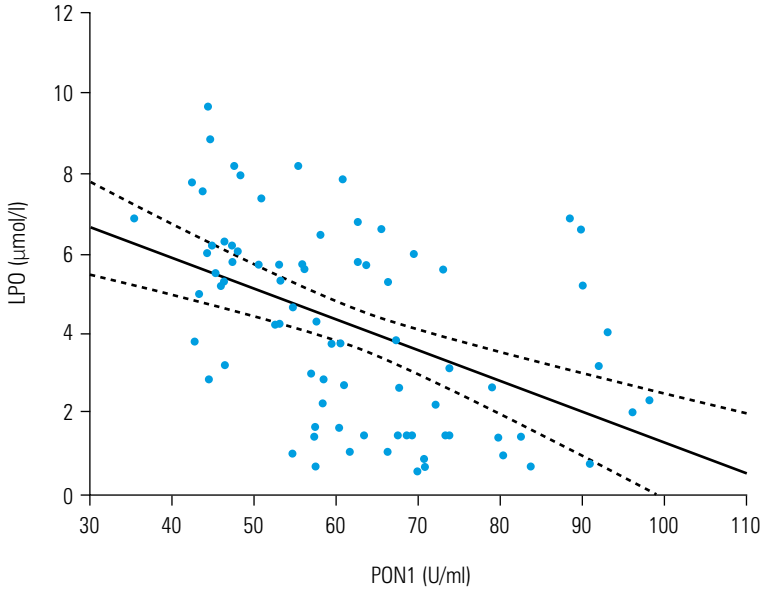

FIGURE 3 A negative correlation between lipid peroxide levels (LPO) and paraoxonase 1 (PON1) activity in patients with type 1 diabetes (n = 80, r = –0.52, $P < 0.05$)

Neither the LPO levels nor the PON1 arylesterase activity correlated with serum levels of metabolic variables. However, a negative correlation between LPO levels and PON1 activity was observed in diabetic patients (n = 80, r = –0.52, $P < 0.05$), as shown in FIGURE 3. Such correlation was not observed in the control group (FIGURE 3).

Late diabetic complications are the main causes of mortality and morbidity in type 1 diabetes patients.[11] Therefore, the metabolic parameters were compared between the groups with and without late diabetic complications. It has been shown that TG levels, postprandial glucose, and HbA_{1c} were higher in patients with complications than in those without late diabetic complications (1.0 [0.7–1.1] vs. 1.2 [0.9–1.3] mmol/l, $P = 0.003$; 7.2 ±0.9 vs. 9.7 ±1.5 mmol/l, $P = 0.003$, 7.5 ±1.6 vs. 8.2 ±1.5%, $P = 0.002$, respectively), as shown in TABLE 2. However, the expected difference for PON1 arylesterase activity and LPO levels was not found.

To establish whether PON1 activity and LPO concentration may be influenced in women by different factors than in men, we compared the parameters measured in patients (TABLE 3) and controls between women and men of the study group. It has been observed that diabetic men had higher TG levels than diabetic women (1.2 [0.8–1.1] vs. 0.7 [0.6–1.1] mmol/l, $P = 0.001$) (TABLE 3).

However, we did not observe any difference between diabetic women and diabetic men for PON1 arylesterase activity and LPO levels. Interestingly, in the subgroup of women a positive correlation between BMI and LPO concentration was observed (n = 28, r = 0.65, $P < 0.05$), as shown in FIGURE 4. This correlation was not observed for diabetic men.

DISCUSSION Although high HDL-C levels observed in the study group brings us to the conclusion that type 1 diabetic patients should be protected against vascular damage,[22] low PON1 activity and high LPO levels found in the study group raise the question of whether this is indeed a fair judgment.

A clinical study proved that low PON1 activity contributes to LPO formation.[23,24] Therefore, it is not surprising that a negative correlation between PON1 activity and LPO levels was observed in the study group. Moreover, low activity of PON1 in the study group suggests that HDL could show functional deficiency in type 1 diabetic patients, despite high HDL-C concentration.[25,26]

However, decreased PON1 activity in patients with type 1 diabetes observed in our study had already been established by other investigators,[27,28] the reason for the enzyme's decreased activity in this patient group is still not fully understood. A possible explanation could be modification of the enzyme's active center resulting from glycation process.[29] Although we did not establish a negative correlation between HbA_{1c} value or glucose levels and PON1 activity, we speculated that lower PON1 activity in type 1 diabetic patients could be the result of chronic hyperglycemia, because most of the patients in the study group had poor metabolic control (HbA_{1c} 8.1 ±1.6%). The study by Mastorikou et al.[30] may indicate that our assumption is valid. They found that isolated HDL from patients with type 2 diabetes showed dramatically lower PON1 activity after in vitro nonenzymatic glycation vs. HDL that was not treated in this process. However, in order to prove that our assumption is correct, an additional study should be conducted that would involve a larger group of patients subdivided into a group with HbA_{1c} ≤7% and >7%.

Low PON1 activity contributes to endothelial cell damage,[31] but it is still unknown whether it is one of the critical factors promoting late diabetic complications in type 1 diabetic patients or not. Some clinical data indicate that low PON1

TABLE 2 Characteristics of patients with type 1 diabetes with and without late diabetic complications

	Patients without complications (n = 44)	Patients with complications (n = 36)	*P*
age, y	31.2 ±5.1	34.0 ±4.2	NS
diabetes duration, y	9.9 ±1.5	10.2 ±1.6	NS
BMI, kg/m^2	22.2 ±4.0	23.6 ±3.0	NS
FPG, mmol/l	8.5 ±2.7	9.05±2.8	NS
PPG, mmol/l	7.2 ±0.9	9.7 ±1.5	0.003
HbA_{1c}, %	7.5 ±1.6	8.2 ±1.5	0.002
TG, mmol/l	1.0 (0.7–1.1)	1.2 (0.9–1.3)	0.003
TC, mmol/l	4.2 (3.0–5.2)	4.7 (3.1–5.6)	NS
LDL-C, mmol/l	2.8 (1.9–3.2)	2.9 (2.1–3.3)	NS
HDL-C, mmol/l	1.6 (1.2–2.0)	1.5 (1.2–1.6)	NS
LPO, µmol/ml	4.5 (1.6–5.5)	4.1 (1.2–5.3)	NS
PON1, U/ml	52.6 (46.0–59.2)	59.3 (42.0–70.0)	NS

Data are given as mean ± SD or median (interquartile range).

Abbreviations: LPO – lipid peroxide, PON1 – paraoxonase 1, others – see TABLE 1

TABLE 3 Characteristics of women and men with type 1 diabetes

	Women (n = 27)	Men (n = 52)	*P*
age, y	34.9 ±7.1	34.1 ±5.9	NS
diabetes duration, y	10.2 ±1.5	9.9 ±1.5	NS
BMI, kg/m^2	23.7 ±3.5	23.2 ±1.2	NS
FPG, mmol/l	8.3 ±2.5	9.0 ±2.0	NS
PPG, mmol/l	8.8 ±2.4	9.0 ±2.1	NS
HbA_{1c}, %	8.2 ±1.6	8.3 ±1.5	NS
TG, mmol/l	0.7 (0.6–1.1)	1.2 (0.8–1.5)	0.001
TC, mmol/l	4.7 (3.0–6.0)	4.7 (4.0–5.2)	NS
LDL-C, mmol/l	2.7 (2.1– 3.6)	2.8 (2.3–3.4)	NS
HDL-C, mmol/l	1.8 (1.6–2.1)	1.5 (1.3–1.8)	NS
LPO, µmol/ml	5.2 (2.8–6.7)	3.8 (1.4–5.8)	NS
PON1, U/ml	57.0 (46.1–70.6)	60.0 (52.6–69.3)	NS

Data are given as mean ± SD or median (interquartile range).

Abbreviations: see TABLES 1 and 2

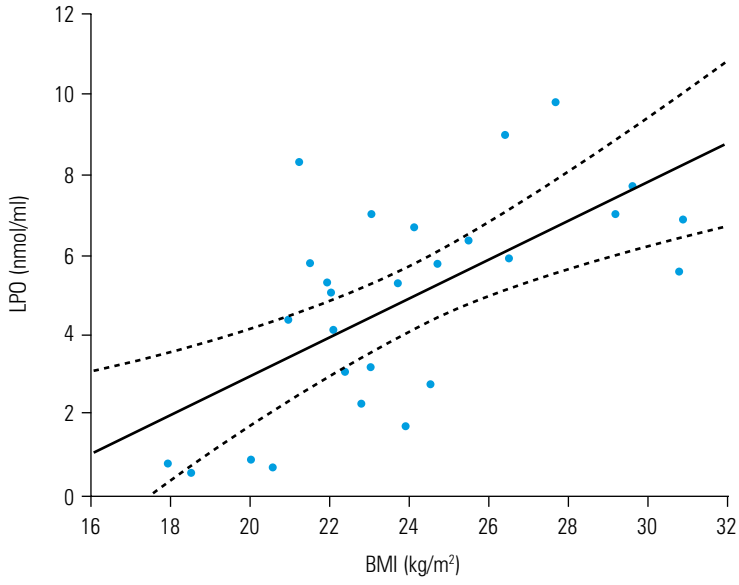

FIGURE 4 Positive correlation between lipid peroxide levels (LPO) and the body mass index (BMI) in women with type 1 diabetes (n = 28, r = 0.65, $P < 0.05$)

contributes to the development of microvascular complications,[32,33] other do not.[14] In addition, there is not much evidence on the association between low PON1 activity and macroangiopathy in patients with type 1 diabetes,[27] although the effect of low PON1 activity on the development of atherosclerosis is better established.[34] In our study, PON1 activity was similar in diabetic patients regardless of the presence or absence of late diabetic complications. Moreover, we found that diabetic patients with and without late diabetic complications did not differ in LPO levels. Therefore, we conclude that low PON1 activity and high LPO levels are not the most critical factors for the development of late diabetic complications in patients with type 1 diabetes.

The analysis of the results concerning LPO levels suggests that enhanced formation of LPO in diabetic patients is related to epidemiological factors. We found that LPO levels are positively associated with high BMI in women with type 1 diabetes, but not in men. However, it has

been established that increased BMI is associated with higher levels of biomarkers of oxidative stress.[35] To our knowledge, the correlation between the BMI and LPO levels had not been observed previously in the subgroup of women with type 1 diabetes. We speculate that it could be associated with different distribution of fat mass in women and men. In men, fat accumulates mainly in the central region, while in women it accumulates in the lower-body/peripheral region. Interestingly, even though both central and peripheral fat deposits are associated with oxidative stress, lower-body fat deposits seem to be more critical for high oxidative stress. Lower-body fat is associated with higher lipoprotein lipase (LPL) activity. It has been established that high LPL activity can contribute to lipid peroxidation.[36] Therefore, our results suggest that enhanced LPO formation in type 1 diabetic women with higher BMI could be associated with oxidative stress. Our assumption is supported by the study of Aslan et al.,[37] who observed a negative correlation between LPO levels and arylesterase PON1 activity and a negative correlation between the BMI and arylesterase PON1 activity in obese patients. It strongly suggests that decreased PON1 activity may be at least partially related to the consumption of PON1 caused by lipid peroxidation process enhanced by obesity.[37]

Moreover, the lack of a negative correlation between LPO levels and BMI in the control group shows that accumulation of fat and enhanced lipid peroxidation process is higher in diabetic women than in healthy women. We think that it could be related to IIT treatment because insulin is an anabolic hormone enhancing lipogenesis,[38] which could indirectly increase LPO formation in this patient group. However, further research is much needed.

Conclusions Low PON1 arylesterase activity suggests insufficient HDL capacity to protect against lipid oxidation in patients with type 1 diabetes. It seems that low PON1 activity and high LPO levels are not the most critical factors for the development of late diabetic complications in patients with type 1 diabetes. Increased LPO formation observed in women with type 1 diabetes seems to be the result of higher lipogenesis, which is not observed in men.

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ARTYKUŁ ORYGINALNY

Ocena aktywności paraoksonazy 1 oraz stężenia nadtlenków lipidowych u chorych na cukrzycę typu 1

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SŁOWA KLUCZOWE

cukrzyca typu 1, nadtlenki lipidowe, paraoksonaza 1, przewlekłe powikłania cukrzycy, stres oksydacyjny

STRESZCZENIE

WPROWADZENIE Niewyrównanie metaboliczne cukrzycy jest jedną z najważniejszych przyczyn rozwoju i progresji przewlekłych powikłań cukrzycowych, ale bada się też inne istotne dla tego procesu czynniki. Zmniejszona aktywność paraoksonazy 1 (PON1) oraz duże stężenie nadtlenków lipidowych (*lipid peroxidation* – LPO) przyczyniają się do uszkodzenia śródbłonka, natomiast nadal nie wyjaśniono, czy istotnie wpływają na rozwój późnych powikłań cukrzycowych.

CELE Celem badania była ocena aktywności aryloesterazowej PON1 i stężenia LPO u pacjentów z cukrzycą typu 1 oraz wyjaśnienie, czy istnieje związek między tymi parametrami a wyrównaniem metabolicznym cukrzycy i rozwojem przewlekłych powikłań cukrzycowych. Ocenialiśmy także, czy wartości PON1 oraz LPO są różne dla kobiet i mężczyzn chorych na cukrzycę typu 1.

PACJENCI I METODY Badaniem objęto 80 pacjentów z cukrzycą typu 1 oraz 24 zdrowe osoby. Aktywność PON1 zmierzono metodą spektofotometryczną, a stężenie LPO oceniono za pomocą komercyjnego testu biochemicznego.

WYNIKI U pacjentów z cukrzycą typu 1 zaobserwowano mniejszą aktywność PON1 oraz większe stężenie LPO niż u osób zdrowych. W grupie badanej wykazano ujemną korelację pomiędzy aktywnością PON1 a stężeniem LPO. Nie znaleziono zależności pomiędzy aktywnością PON1 i stężeniem LPO a wyrównaniem metabolicznym cukrzycy lub występowaniem przewlekłych powikłań cukrzycy. Natomiast u kobiet z cukrzycą typu 1 wykazano dodatnią korelację pomiędzy stężeniem LPO a wartością wskaźnika masy ciała.

WNIOSKI Wyniki naszych badań wskazują, że zmniejszona aktywność PON1 oraz duże stężenie LPO nie są najważniejszymi czynnikami wpływającymi na rozwój przewlekłych powikłań w cukrzycy typu 1. Natomiast zaobserwowane duże stężenie LPO u kobiet z cukrzycą typu 1 może być wynikiem przyspieszonej lipogenezy w tej podgrupie w porównaniu z mężczyznami z cukrzycą typu 1.

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