Paraoxonase and arylesterase levels in autoimmune thyroid diseases

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Objective: The aim of this study was to evaluate serum paraoxonase-1 (PON1) activity and its association with oxidative stress in autoimmune thyroid disease (AITD).
Methods: A total of 50 patients with AITD, including 25 with Hashimoto's thyroiditis and 25 with Graves' disease were enrolled. The control group comprised 27 healthy subjects. Blood samples were obtained in the euthyroid period and 3 months after initiation of medical treatment. Serum samples from patients with AITD and the healthy control group were analyzed for basal PON1, salt-stimulated PON1, and arylesterase (ARE) activities, along with lipid hydroperoxide (LOOH) and total free sulfhydryl (–SH) levels.
Results: Serum PON1 activities and –SH levels were significantly lower ($P < 0.001$, for each), whereas LOOH levels were significantly higher ($P < 0.001$, for each) in patients with AITD, compared to the control group. We observed no significant differences in ARE levels between the patient and healthy control groups ($P > 0.05$). PON1 activity was positively correlated with –SH ($r = 0.522$, $P < 0.001$) and negatively correlated with LOOH ($r = -0.487$, $P < 0.001$). PON1 phenotype distribution of the subjects was not significantly different among the three groups ($P = 0.961$).
Conclusions: Serum PON1 activity is decreased in patients with AITD, and correlated positively with –SH, a well-known antioxidant, and negatively with LOOH, an index of lipid oxidation.

Keywords: Arylesterase, Autoimmune thyroid disease, Oxidative status, Paraoxonase

Introduction

Oxidation of iodide to iodine is an important step for thyroid hormone synthesis. This reaction is catalyzed by thyroid peroxidase and involves hydrogen peroxide (H_2O_2) and oxidized iodine. Therefore, a certain oxidative load level in the thyroid gland is required for thyroid hormone synthesis. Strong antioxidant systems exist to protect thyroid cells against increased levels of oxidants, such as glutathione peroxidase, peroxiredoxin, and catalase.[1,2]

Oxidative stress occurs when production of reactive oxidant species (ROS) is not controlled by antioxidant systems. Excessive oxidative stress increases inflammation, causes proapoptotic effects, and impairs immune tolerance, thereby contributing to the pathogenesis of various autoimmune diseases, including autoimmune thyroid disease (AITD).[3,4]

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Lipid hydroperoxide (LOOH) is a well-known marker of oxidative stress. The basic form of LOOH is the oxidized low-density lipoprotein (LDL).[5,6] Increased levels of lipid peroxidation products have been reported in AITD.[7,8]

Paraoxonase1 (PON1) is an antioxidant enzyme with paraoxonase (PON) and arylesterase (ARE) activities. PON1 protects LDL from oxidation by binding to high-density lipoprotein (HDL).[9] PON1 activity has been shown to be reduced in several autoimmune diseases, including rheumatoid arthritis (RA), mixed connective tissue disease, Sjögren's syndrome, and systemic lupus erythematosus (SLE).[10,11,12,13] However, PON1 activity in AITD has not been investigated to date. In the present study, we evaluated PON1 activity in AITD and its association with antioxidant total free sulfhydryl (–SH) and oxidant LOOH levels.

Materials and methods

The study was approved by the Ethics Committee of Gaziantep University Faculty of Medicine. Informed

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consent was obtained from all subjects prior to the study.

Patient groups and study protocols

This prospective study was conducted in the Department of Endocrinology at the Medical School of Gaziantep University. A total of 50 patients with autoimmune thyroid disease, including 25 with Graves' disease (GD) (mean age: 44.00 ± 14.36 years; 17 females, 8 males) and, 25 with Hashimoto's thyroiditis (HT) (mean age: 43.92 ± 12.78 years; 16 females, 9 males) as well as 27 healthy controls (mean age: 41.44 ± 9.53 years; 16 females, 11 males) were enrolled.

Thyroid ultrasonography was performed in all patients by the same physician, regardless of laboratory results (LOGIQ p6, GE Healthcare, USA). Size, nodularity, and paranchymal echogenicity of the thyroid gland were evaluated.

Diagnosis of HT was based on thyroid function tests (low/normal serum free triiodothyronine (T3) and free thyroxine (T4), high thyroid stimulating hormone (TSH) levels), heterogeneous appearance of the thyroid parenchyma in ultrasound, and increased levels of antithyroid peroxidase (anti-TPO) and antithyroid thyroglobulin (anti-TG) antibodies. Levothyroxine therapy was initiated in these patients. Serum samples were obtained during the euthyroid period in month 3 of the treatment.

Diagnosis of GD was based on thyroid function tests (high/normal serum free triiodothyronine (T3) and free thyroxine (T4), low TSH levels), increased levels of TSH receptor antibody (anti-TRAB), anti-TPO, and anti-TG antibodies, heterogeneous appearance of the thyroid parenchyma in thyroid ultrasound, increased diffuse radioactive iodine uptake in thyroid scintigraphy, and the medical history of the patients. Methimazol therapy was initiated in these cases. Serum samples were obtained during the euthyroid period in month 3 of treatment.

Subjects had infectious diseases, inflammatory diseases, hypertension, liver failure, cardiovascular diseases, malignancies, neurodegenerative diseases, renal failure, cerebrovascular diseases, diabetes mellitus, obesity, and metabolic syndrome. Patients from both study and control groups on antioxidant treatments, such as antihypertensive and, lipid-lowering medications, and vitamin E, and smokers were excluded.

Measurements

Age, weight, height, and body mass index (BMI: body weight (kg)/height (m^2)) of all subjects were recorded. Blood samples were collected in the morning after an 8 hours fasting period. Serum samples were stored at −80°C until measurement of PON, ARE, –SH, and LOOH. Fasting plasma glucose (FPG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), total cholesterol, triglyceride (TG), free T3, free T4, TSH, anti-TPO, and anti-TG levels were measured in all subjects.

Free T3, free T4, and TSH levels were measured with the electrochemiluminescence method, using a Cobas Integra 800 model auto-analyzer. The reported reference ranges were as follows: free T3: 2–4, 4 pg/ml, free T4: 0.7–1.48 ng/dl, TSH: 0.35–4.94 uIU/ml (Roche Diagnostics, Germany).

Measurement of LOOH levels

Serum LOOH levels were measured with the ferrous ion oxidation–xylenol orange assay. The principle of the assay depends on oxidation of the ferrous to ferric ion through various oxidants, and the produced ferric ion is measured with xylenol orange. LOOH is reduced by triphenyl phosphine (TPP), a specific reductant for lipids. LOOH levels are determined from the difference with and without TPP pretreatment.[14]

Measurement of serum –SH levels

Free sulfhydryl serum levels were measured using the method of Ellman.[15] For determination of –SH groups, 1 ml of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 50 µl serum were added into cuvettes, followed by 50 µl of 10 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in methanol. Reagent blanks were run for each sample, substituting methanol alone for DTNB in methanol. Following incubation for 15 minutes at room temperature, sample absorbance was read at 412 nm on a Cecil 3000 spectrophotometer. Sample and reagent blanks were subtracted. Sulfhydryl group concentrations were calculated using reduced glutathione as the free sulfhydryl group standard, and the results expressed as millimolar (mM).

PON1 and ARE activities

PON1 and ARE activities were measured with commercially available kits (Relassay, Gaziantep, Turkey). PON1 measurement was performed either in the presence (salt-stimulated) or absence of NaCl. The paraoxon hydrolysis rate (diethyl-*p*-nitrophenyl phosphate) was measured by monitoring increased absorption at 412 nm at 37°C. The amount of *p*-nitrophenol generated was calculated from the molar absorption coefficient at pH 8.5, which was 18.290/M/cm.[16] PON1 activity was expressed as U/l serum. The coefficient of variation (CV) for individual samples was 1.8%. ARE activity was measured using phenyl acetate as the substrate. Enzymatic activity was calculated from the molar absorption coefficient of the phenol produced, 1310/M/cm. One unit of ARE activity was defined as 1 mmol phenol generated per minute under the above conditions, and expressed

as U/l.[17] The CV for individual serum samples was 4.1%. Sensitivities of both tests were over 98%.

PON1–Q192R phenotyping

PON1–Q192R phenotype distributions were determined using both paraoxon and phenylacetate as substrates according to the method of Eckerson *et al*.[18] To determine the phenotype of a given participant as QQ (homozygous low activity) or QR (heterozygous moderate activity), the ratio of salt-stimulated PON1 to ARE activity was used to assign phenotypes to individuals. After frequency analysis, subjects with salt-stimulated PON1 to ARE ratio <2.90 were classified as the homozygous AA group (PON1–192QQ; $n = 21$ for GD, $n = 21$ for HT, $n = 22$ for healthy control), those with ratios between 2.90 and 5.07 as the heterozygous AB group (PON1–192QR; $n = 4$ for GD, $n = 4$ for HT, $n = 5$ for healthy control), and those with ratios >5.08 (PON1–192RR; $n = 0$ for each) as the homozygous BB group.

Statistical analysis

The Shapiro-Wilk test was used to test continuous variables for normality. Measurements of normally distributed variables (BMI, FPG, total-C, HDL-C, LDL-C, TG, free T3, free T4, TSH) are presented as mean ± standard deviation. Variables with non-normal distribution (PON1, ARE, LOOH, –SH) are presented as median and interquartile range (IQR). Student's *t*-test was used to compare two independent groups of normally distributed variables. For non-normally distributed variables, the Mann–Whitney *U* test was used to compare two independent groups. Spearman correlation analysis was conducted to identify the associations between the parameters. The statistical significance of differences in phenotype frequency between the groups was tested using the chi-square (χ^2) test. SPSS for Windows version 15 software was applied for statistical analyses. The level of significance was set at $P \leq 0.05$.

Results

The mean ages of the GD, HT, and healthy control groups and gender distribution were similar ($P = 0.970$ and $P = 0.806$, respectively). BMI, free T3, free T4, TSH, FPG, total cholesterol, HDL-C, LDL-C, and TG levels were not significantly different among the groups ($P = 0.363$, $P = 0.208$, $P = 0.825$, $P = 0.400$, $P = 0.785$, $P = 0.325$, $P = 0.491$, $P = 0.070$, $P = 0.295$, respectively; Table 1).

Serum PON1 activity was markedly lower in the GD and HT groups, compared to the healthy control group ($P < 0.001$). PON1 activity was not significantly different between the GD and healthy control groups ($P = 0.143$). Serum salt-stimulated PON1 activities were significantly lower in patients, compared to the healthy control group ($P = 0.035$ for GD, $P = 0.024$ for HT), whereas serum ARE levels were not significantly different among the groups ($P > 0.05$).

PON1 phenotype distribution of the subjects was not notably different among the three groups ($P = 0.961$).

Serum LOOH levels were significantly higher in the GD and HT groups, compared to the healthy control group ($P < 0.001$, for each). Moreover, the HT group displayed markedly higher LOOH levels than the GD group ($P = 0.008$).

Table 1 Demographic and laboratory parameters of HT, GD, and healthy control groups

	GD	HT	Healthy controls	*P*
Age (year)	44.00 ± 14.36	41.44 ± 9.53	43.92 ± 12.78	0.811
Gender (F/M)	17/8	16/11	16/9	0.806
BMI (kg/m^2)	25.59 ± 3.06	26.64 ± 2.16	25.78 ± 2.01	0.375
FBG (mg/dl)	87.72 ± 6.47	86.96 ± 6.47	88.92 ± 8.15	0.760
Total-C (mg/dl)	175.00 ± 30.44	179.04 ± 30.53	173.48 ± 28.64	0.889
HDL-C (mg/dl)	47.92 ± 6.71	47.68 ± 6.59	46.74 ± 6.34	0.558
LDL-C (mg/dl)	112.44 ± 22.64	116.04 ± 29.26	105.25 ± 25.30	0.354
TG (mg/dl)	130.00 ± 29.32	129.80 ± 26.94	137.85 ± 46.26	0.624
Free T3 (pg/ml)	2.54 ± 0.38	2.82 ± 0.52	2.69 ± 0.48	0.075
Free T4 (ng/dl)	1.14 ± 0.23	1.08 ± 0.17	1.06 ± 0.14	0.189
TSH (uIU/ml)	2.15 ± 0.52	2.05 ± 0.99	2.29 ± 0.59	0.541
PON1 (U/ml)*	106.32 ± 14.38[a]	92.15 ± 28.75[b]	125.45 ± 26.18	<0.001
Salt-stimulated PON (U/ml)*	175.05 ± 102.27	161.23 ± 116.93	215.76 ± 93.76	0.035
ARE (U/ml)*	101.09 ± 48.51	109.47 ± 25.49	102.07 ± 34.69	0.241
LOOH (mol H_2O_2 equiv./l)*	11.30 ± 4.88[a,c]	13.11 ± 4.07[b]	5.98 ± 3.08	<0.001
–SH (mmol/l)*	0.16 ± 0.05[a]	0.18 ± 0.09[b]	0.45 ± 0.12	<0.001

Data were expressed as mean ± SS and *median (IQR). $P < 0.005$ was regarded as statistically significant. Graves' disease, GD; Hashimoto thyoriditis, HT; body mass index, BMI; fasting blood glucose, FBG; high-density lipoprotein-cholesterol, HDL-C; low-density lipoprotein-cholesterol, LDL-C; triglyceride, TG; triiodothyronine, T3; tetraiodothyronine, T4; thyroid stimulating hormone, TSH; lipid hydroperoxide, LOOH; paraoxonase-1, PON1; arylesterase, ARE; total free sulfhydryl, –SH.
[a]$P<0.001$ GD versus healthy control.
[b]$P<0.001$ HT versus healthy control.
[c]$P<0.01$ GD versus HT.

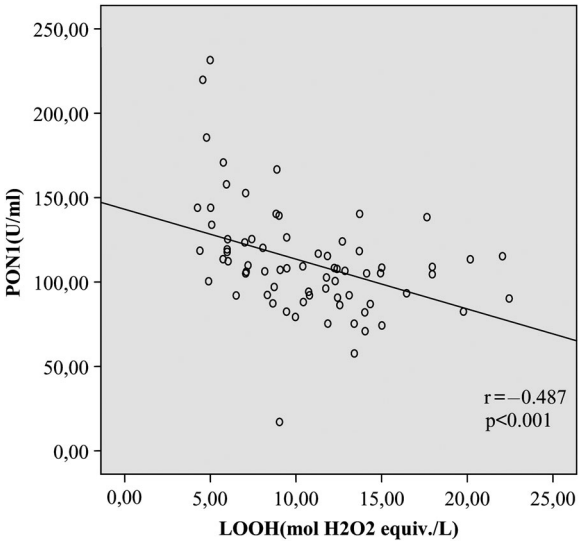

Figure 1 Correlation between serum basal PON1 and LOOH levels.

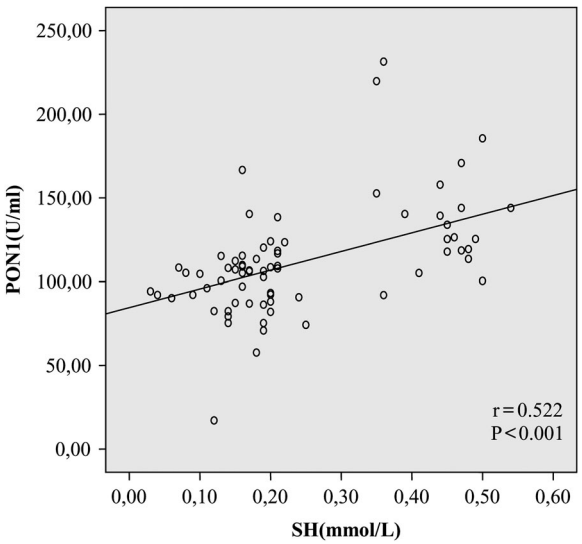

Figure 2 Correlation between serum basal PON1 levels and –SH.

Serum –SH levels were significantly lower in the GD and HT groups, compared to the healthy control group ($P < 0.001$, for each). We observed no notable differences between the GD and HT groups in terms of –SH levels ($P = 0.869$).

In correlation analysis, PON1 activity was positively correlated with –SH ($r = 0.522$, $P < 0.001$) and negatively correlated with LOOH in patients with AITD ($r = -0.487$, $P < 0.001$; Figs. 1 and 2).

Discussion

In our experiments, PON1 activity was significantly reduced in AITD patients, compared to the control group ($P < 0.001$). Additionally, PON1 activity was positively correlated with the antioxidant –SH and negatively correlated with the oxidant LOOH ($r = 0.522$, $P < 0.001$, $r = -0.487$, $P < 0.001$). To our knowledge, the present study is the first to report reduced PON1 activity in AITD.

H_2O_2 is required for thyroid hormone biosynthesis in thyrocytes.[19] Thyroglobulin fragmentation is observed upon exposure of thyroid cell culture to high concentrations of H_2O_2. Extended exposure of thyrocytes to H_2O_2 and/or impaired antioxidant systems are suggested to alter the antigenicity of thyroglobulin and thyroid peroxidase, in turn, contributing to AITD development.[20]

Lipids are the most sensitive molecules to the effects of free radicals. Free radicals react with polyunsaturated fatty acids in the cell membrane, causing oxidative degradation known as lipid peroxidation, a process of self-sustaining chain reactions.[21,22] A number of studies in the literature have demonstrated increased lipid peroxidation products in AITD.[4,7]

LOOH, a product of lipid peroxidation, is used as an indicator of the lipid peroxidation level.[21,23] Data from the present study revealed increased LOOH levels in AITD patients and decreased levels of the antioxidant –SH ($P < 0.001$, for each), compared to healthy controls.

PON1 contributes significantly to the antioxidant capacity of HDL.[23] Activity of the enzymes reduced in several autoimmune diseases, such as RA, mixed connective tissue disease, SLE, and primary antiphospholipid syndrome.[10,11,13] To date, PON1 activity has not been evaluated in AITD. Here, we observed significantly lower PON1 activity in AITD patients, compared to the healthy control group ($P < 0.001$). The comparable lipid levels among the groups in our study suggest that reduction of PON1 in AITD occurs independently of HDL. We propose that the reduced levels of PON1 may be attributable to use of the enzyme by thyrocytes for protection from increased oxidative stress. A study by Deakin *et al.* on hamster ovaries and human endothelial cells demonstrated that PON1 passes through the cell membrane and outer environment easily, reduces oxidative stress of the target cell to a significant extent, and facilitates cells to gain function. The group suggested that PON1 is not fixed to HDL and may be used by cells exposed to oxidative stress.[24]

PON1 levels in our study were significantly lower in AITD, compared to the healthy control group, whereas no differences among groups were observed in ARE levels. Previously, we showed reduced PON activity in Sjögren's syndrome, compared to the control group, but no differences in ARE activity, which was suggested to result from PON1 gene polymorphisms.[12] PON1 activity was higher in individuals with the Q192R polymorphism resulting in a glutamine to arginine substitution at position 192, whereas ARE activity was higher in the QQ genotype.[25] The ratio of salt-stimulated PON1 to ARE

activity is used to define phenotypes.[26] We observed no significant differences between PON1 192Q and R polymorphism distribution among the GD, HT, and healthy control groups. Based on these data, we propose that the use of PON1 to prevent oxidative stress-induced thyroid cell damage is the underlying cause of low PON1 activity.

In conclusion, serum activity of PON1 is decreased in AITD. Additionally, reduced activity of PON1 is positively correlated with levels of the antioxidant, –SH, and negatively correlated with the oxidant, LOOH. Our results collectively suggest that PON1 contributes to the pathogenesis of AITD. Extensive longer-term studies are required to validate the current findings.

Disclaimer statements

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Conflicts of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethics approval None.

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