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Effects of caloric restriction and gender on rat serum paraoxonase 1 activity☆

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Abstract

Paraoxonase 1 (PON1) associates to specific high-density lipoproteins (HDLs) — those containing apolipoprotein A-I (apoA-I) and apolipoprotein J (apoJ) — and is largely responsible for their antiatherogenic properties. Caloric restriction (CR) has been shown to reduce major atherosclerotic risk factors. The aims of this work were to study PON1 activity response to CR (40% over 14 weeks) and to elucidate whether there are adaptive differences related to gender. Serum and liver paraoxonase and arylesterase activities, serum triglyceride, total and HDL cholesterol concentrations, serum PON1, apoA-I and apoJ contents and liver PON1 mRNA levels were measured. No effects of CR or gender were observed in triglyceride, total cholesterol concentration and PON1 mRNA levels. HDL cholesterol was higher in female rats than in male rats and increased with CR only in the latter animals. Serum PON1 activities tended to be higher in female rats and dropped with CR, with females showing the biggest decrease. Serum PON1 content was higher in female rats and decreased in both genders with CR, whereas apoA-I and apoJ contents, which were higher in female rats too, decreased only in the former animals, accounting for the high PON1 activity decrease observed in these animals. In conclusion, the short-term CR-associated reduction of serum PON1 activity and PON1, apoA-I and apoJ levels points toward a reduced stability of HDL–PON1 complexes and/or HDL particle levels responsible for PON1 transport and function in the blood. Moreover, the variations in PON1 activity and apolipoprotein levels show gender-related differences that are indicative of a different adaptive strategy of male and female rats when faced with a period of food restriction.

1. Introduction

High-density lipoproteins (HDLs) have long been known to be antiatherogenic [1], and their protective role has been in part attributed to their associated enzymes [2]. Of these, paraoxonase 1 (PON1) — an esterase synthesized and stored mainly by the liver and with a broad substrate specificity [3] — has been shown to be largely responsible for the antioxidant properties of HDL. Serum PON1 activity has been shown to be inversely related to the risk of cardiovascular diseases [4]. PON1 limits the accumulation of lipid oxidation products in low-density lipoproteins (LDLs) and HDLs, prevents the transformation of LDLs into proatherogenic particles and reverses the biologic effects of oxidized LDLs [5–9], thus attenuating the initiation and progression of atherosclerotic lesions.

PON1 mainly associates to specific HDL particles containing apolipoprotein A-I (apoA-I) without apoA-II [10,11]. apoA-I, although not required for the association of PON1 with HDL particles, stabilizes HDL–PON1 complexes [12,13]. Indeed, an important part of PON1 activity has been found in HDLs also containing apolipoprotein J (apoJ) [10,14], which is capable of reducing the oxidative potential of LDLs by itself [15], thus dovetailing with the antioxidant role of PON1.

Dietary and lifestyle factors, which are promoters of cardiovascular disease, have been shown to modify PON1 activity and/or concentration [16–18]. Proatherogenic diets are related to a significant drop in PON1 activity and/or concentration [19–21]. Since PON1 is highly susceptible to inactivation by oxidation [22], caloric

Abbreviations: apoA-I, apolipoprotein A-I; apoJ, apolipoprotein J; CR, caloric restriction; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PON1, paraoxonase 1; 18S, 18S ribosomal RNA.

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restriction (CR) — which has been related to a lower oxidative status as well as to beneficial effects on major atherosclerosis risk factors [23] — would be an adequate experimental model to study PON1.

Thus, the aims of this work were to study PON1 activity response to CR (40%) and to elucidate whether there are adaptive differences related to gender. To go further into the factors involved in the modulation of PON1 activity by CR, we studied serum levels of PON1 and apolipoproteins related to serum PON1 stability and function, as well as liver PON1 activity and gene expression. According to our results, we reached the conclusion that CR decreases serum PON1 activity through a reduction in the stability of HDL–PON1 complexes and/or the number of HDLs containing PON1 in the blood and that this response to CR is gender dependent.

2. Materials and methods

2.1. Materials

Oligonucleotide primer sequences, Lightcycler-FastStart DNA Master SYBR Green I for real-time PCR and Tripure isolation reagent were purchased from Roche Diagnostics (Basel, Switzerland). RT-PCR chemicals were from Applied Biosystems (CA, USA). Rabbit polyclonal antibody to human PON1 was kindly provided by Dr. M.I. Mackness and Dr. B. Mackness [24]. Rabbit polyclonal antibody to human apoA-I was supplied by Calbiochem (Cat. Num. 178422, San Diego, CA, USA); goat polyclonal antibody to rat apoJ, by Santa Cruz Biotechnology (Cat. Num. sc-13747, Santa Cruz, CA, USA). A chemiluminescence kit (ECL) for immunoblot development was purchased from Amersham (Little Chalfont, UK). Kits for serum lipid profile measurement were purchased from Linear Chemicals (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA). Substrates for the measurements of PON1 activities (diethyl p-nitrophenyl phosphate and phenylacetate) were from Sigma-Aldrich. Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain), Sigma-Aldrich and Roche Diagnostics.

2.2. Animals and experimental protocols

Animal experiments were performed in accordance to general guidelines approved by our institutional ethics committee and EU (86/609/EEC) regulations. Eight-week-old rats (Charles River, Barcelona, Spain), six males and six females, were fed ad libitum with a pelleted standard diet (A04, Panlab, Barcelona, Spain). The composition of the diet was as follows: 2.9% fat, 15.4% protein, 60.5% carbohydrates, 3.9% fiber, 5.3% minerals and 12% humidity (3000 kcal/kg). The same number of animals daily received 60% of the food intake of the control animals (40% energy restriction), with free access to water over 14 weeks. The amount of food offered to CR animals was updated weekly according to the intake of the control rats. Rats were housed individually in wire-bottom cages to prevent coprophagia at 22°C with a 12-h light/dark cycle.

2.3. Sample collection and measurement of serum lipid profile

Animals were killed by decapitation at the beginning of the light cycle. Livers were immediately removed, frozen in liquid nitrogen and stored at −70°C until used for microsomal fraction and total RNA isolation. Serum samples were stored at −70°C until analyzed.

HDL cholesterol levels were measured by using an enzymatic homogeneous assay (HDL cholesterol direct–Cat. Num. 1133505). Total cholesterol and triglyceride levels were measured by using spectrophotometric assay kits (Total cholesterol–Cat. Num. 1118005 and TNT 10−Cat. Num. 336-10, respectively).

2.4. Isolation of liver microsomal fraction

Microsomal isolation was performed as previously described [25] but with minor modifications. Briefly, a small piece of liver was minced with scissors and, finally, homogenized in a homogenization buffer (5-mmol/L Tris–HCl buffer, pH 7.4, containing 0.25-mol/L sucrose) using a Potter-Elvehjem. The homogenates were filtered, and nuclei and mitochondria were removed by successive centrifugation at 460×*g* and 12,500×*g* for 10 min, respectively, in a refrigerated centrifuge (SIGMA 3K30). The postmitochondrial supernatant fraction was then centrifuged at 100,000×*g* for 1 h in a refrigerated ultracentrifuge (BECKMAN L7-55) to obtain the microsomal fraction. Microsomal pellets were resuspended in a 5-mmol/L Tris–HCl buffer (pH 7.4) and immediately used for enzymatic assays. Aliquots of microsomal fraction were stored at −70°C for total protein content determination [26].

2.5. Enzyme assay

Arylesterase (EC 3.1.8.1) and paraoxonase (EC 3.1.1.2) activities were assayed in serum and liver microsomal samples in a microtiter plate spectrophotometer (Bio-Tek Instruments, VT, USA). Arylesterase and paraoxonase activities were assayed by measuring the rate of hydrolysis of phenylacetate and paraoxon (diethyl p-nitrophenyl phosphate), respectively [27].

2.6. Analysis of PON1 mRNA levels by real-time RT-PCR

Total cellular RNA was isolated from liver samples by using a Tripure isolation reagent according to the manufacturer's protocol.

One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 1 h, with 25 U of MuLV RT in a 10-μl volume of RT reaction mixture containing 10-mmol/L Tris–HCl (pH 9.0), 50-mmol/L KCl, 0.1% TritonX-100, 2.5-mmol/L $MgCl_2$, 2.5-μmol/L random hexamers, 10-U RNase inhibitor and 500-μmol/L dNTP (each). cDNA samples were diluted 1/10, and aliquots were frozen at −70°C until the PCRs were carried out.

Real-time PCR was performed using SYBR Green detection technology in a Lightcycler Rapid Thermal Cycler (Roche Diagnostics). Each reaction contained 1 μl of Lightcycler-FastStart DNA Master SYBR Green I (containing FastStart Taq DNA polymerase, dNTP mix, reaction buffer, $MgCl_2$ and SYBR Green I dye; Roche Diagnostics), 0.5 μmol/L of each specific primer, 2 mmol/L of $MgCl_2$ and 3 μl of the cDNA dilution in 10 μl of the total reaction volume.

Oligonucleotide primer sequences used for real-time PCR were 5′-tgggcctgtcatggtccaatgttg-3′ and 5′-ttatccacaagggtgtcaaagctgag-3′ for PON1 and 5′-gaggtgaaattcttggaccgg-3′ and 5′-cgaacctccgactttcgttct-3′ for 18S ribosomal RNA (18S), which was used as housekeeping. For each oligonucleotide primer sequence, a basic local alignment search tool (NCBI, BLAST) revealed that an optimum sequence homology was obtained for the target gene.

The amplification programs were as follows: a preincubation step for denaturation of template cDNA at 95°C for 10 min, followed by 40 cycles consisting of a denaturation step (95°C for 15 s for PON1 and 95°C for 5 s for 18S), an annealing step (60°C for 1 min for PON1 and 60°C for 8 s for 18S) and, finally, an extension step (72°C for 12 s). Melting programs were 95°C for 5 s, 65°C for 15 s and 99°C for 0 s.

Real-time PCR efficiencies were calculated on average of efficiencies from each sample, which were calculated by using the following formula: $e=(F/F_0)^{1/(n-n_0)}$, where F and F_0 mean fluorescence values belonging to the linear segment of each PCR quantification curve and n and n_0 represent their corresponding crossing points. PON1 and 18S real-time PCR efficiencies were 1.89 and 1.87, respectively. Melting temperatures of PON1 and 18S were 82°C and 83°C, respectively. PCR product sizes were 246 pb for PON1 and 93 pb for 18S.

2.7. Western blot analysis of serum PON1, apoA-I and apoJ protein levels

Equal amounts of serum protein (30 μg) were fractionated on 15% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Ponceau S staining was performed systematically to check the correct loading and electrophoretic transfer. Rabbit polyclonal antibodies against human apoA-I and PON1 and goat polyclonal antibodies against rat apoJ were used as primary antibodies. Anti-rabbit and anti-goat IgG-alkaline phosphatase antibodies were used as secondary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). The apparent molecular weights of apoA-I (28 kDa), PON1 (43 kDa) and apoJ (50 kDa) were estimated using protein molecular mass standards.

2.8. Statistical analysis

All data are expressed as the mean values±S.E.M. of six animals per group. Statistical analyses were performed by using a statistical software package (SPSS 11.0 for Windows, SPSS, Chicago, IL, USA). Differences between experimental groups in biochemical parameters were assessed by two-way analysis of variance (ANOVA) followed by Student's t test, as post hoc comparison, only when an interactive effect of CR and gender was shown. A P value $<.05$ was considered statistically significant.

The statistical PCR data analysis was performed by using the Relative Expression Software Tool. Differences in mRNA levels between groups were analyzed with the Pairwise Fixed Reallocation Randomization Test [28], a proper model to avoid the normal distribution of data.

3. Results

3.1. Biometrical parameters and serum lipid profile

A 14-week period of CR induced a 27% decrease in the body weight of the male and female rats ($P<.001$; Table 1). No effects of CR or gender were observed in the relative weight of the livers. Microsomal protein concentration in CR rats did not differ from that of control rats; however, male rats had a higher microsomal protein concentration than female rats. Serum triacylglyceride and total cholesterol concentration remained at the same level in all

Table 1
Biometrical parameters and serum lipid profiles

	Male		Female		ANOVA ($P<.05$)
	Control	CR	Control	CR	
Body weight (g)	482±14	354±8[a]	261±8[b]	194±4[a, b]	G, CR, G*CR
Relative liver weight (g/100 g)	2.84±0.12	2.77±0.11	2.75±0.12	2.91±0.13	NS
Liver microsomal protein (mg/g)	11.5±1.6	12.0±1.8	7.67±0.49	10.9±3.0	G
Triglyceride (mmol/L)	18.4±2.2	16.3±1.3	12.4±1.8	15.2±2.0	NS
Total cholesterol (mmol/L)	1.24±0.09	1.37±0.09	1.13±0.07	1.28±0.15	NS
HDL cholesterol (mmol/L)	0.490±0.095	0.677±0.086[a]	0.701±0.063[b]	0.623±0.097	G*CR

Values are expressed as the mean±S.E.M. of six animals per group. G indicates gender effect; CR, CR effect; G*R, interactive effect; NS, nonsignificant. t Test: $P<.05$.

[a] CR vs. control.

[b] Female vs. male.

Table 2
Effect of CR on serum and liver paraoxonase and arylesterase activities in male and female rats

	Male		Female		ANOVA ($P<.05$)
	Control	CR	Control	CR	
Serum					
Paraoxonase activity (mUI/ml)	42.5±2.9	31.6±1.5	53.5±2.2	29.2±3.1	CR
Arylesterase activity (UI/ml)	42.9±3.0	35.7±2.2	52.7±3.7	31.4±5.5	CR
Liver					
Paraoxonase activity (mUI/mg protein)	1.48±0.20	1.80±0.18	1.10±0.17	1.77±0.19	CR
Arylesterase activity (UI/mg protein)	6.37±1.39	7.16±1.77	8.70±1.07	6.53±0.96	NS

Values are expressed as the mean±S.E.M. of six animals per group.

experimental groups. Control male rats had a lower HDL cholesterol concentration than female rats, and CR increased HDL cholesterol concentration in male rats but not in female rats.

3.2. Effect of CR on serum PON1 activity and content and liver PON1 activity and mRNA levels

PON1 activity was measured in both serum and liver microsomal fraction samples of control and CR rats (Table 2). After the period of CR, male and female rats underwent a 26% and a 46% decrease of serum paraoxonase activity, respectively ($P<.001$). Serum arylesterase activity decreased by 17% and 41% in male and female CR rats, respectively, as compared with control rats ($P<.05$). Therefore, the decrease of both activities in response to CR was higher in female rats compared with male rats. Control female rats showed slightly higher serum paraoxonase and arylesterase activities (28% and 23%, respectively) than control male rats, although differences did not reach statistical significance ($P=.069$ and $P=.124$ for paraoxonase and arylesterase activities, respectively). CR caused an increase in liver paraoxonase activity ($P=.015$), and, although there was no gender effect, the increase was more important in female rats (22% for male rats and 61% for female rats). However, CR did not modify arylesterase activity.

Serum PON1 content was also measured in all experimental groups (Fig. 1). PON1 serum levels were significantly higher in female rats and decreased by approximately 35% with CR in male and female rats. We found strong positive correlations between serum PON1 levels and serum paraoxonase and arylesterase activities ($R=.598$, $P=.007$ and $R=.531$, $P=.019$, respectively).

Liver PON1 mRNA levels were also measured, since serum PON1 is mainly synthesized in this tissue. Liver PON1 mRNA levels were not modified by CR or gender (100±2% for control male rats, which was taken as reference, 98.3±2.03% for CR male rats, 97.4±3.0% for control female rats and 98.6±1.0% for CR female rats). PON1 mRNA levels strongly correlated with liver arylesterase activity ($R=.524$, $P=.015$).

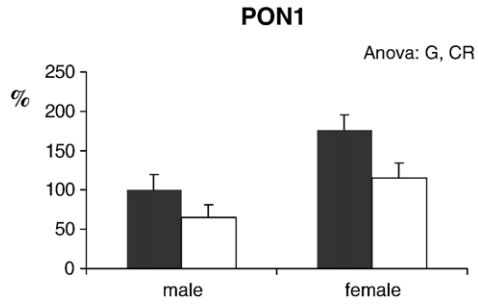

Fig. 1. Effect of CR on PON1 serum levels in male and female rats. PON1 levels of control male rats were set as 100%. Data represent the mean±S.E.M. of six animals. ANOVA ($P<.05$): G indicates gender effect; CR, CR effect. Control and CR groups are represented by black and white bars, respectively.

3.3. Effect of CR on apoA-I and apoJ serum levels

Serum apoA-I levels, a suitable marker of the total HDL population [29], were measured in all experimental groups (Fig. 2A). Furthermore, serum apoJ levels, a direct marker of the HDL subfraction that carries PON1, were also determined (Fig. 2B). Control female rats showed higher serum apoA-I and apoJ levels than male rats, although only apoA-I reached statistical significance ($P<.05$). In female rats, CR caused a marked decrease in both serum apoA-I (64%) and apoJ (42%) levels, whereas in male rats, no

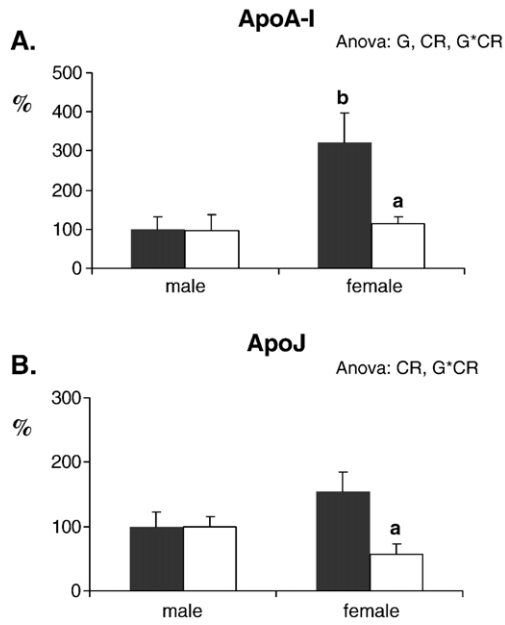

Fig. 2. Effect of CR on apoA-I (A) and apoJ (B) serum levels in male and female rats. Apolipoprotein levels of control male rats were set as 100%. Data represent the mean±S.E.M. of six animals. ANOVA ($P<.05$): G*CR indicates an interactive effect. t Test ($P<.05$): Superscript letters indicate CR vs. control (a) and female vs. male (b). Control and CR groups are represented by black and white bars, respectively.

Table 3
Effect of CR on apoA-I/HDL cholesterol, apoJ/HDL cholesterol and PON1/HDL cholesterol ratios of male and female rats

	Male		Female		ANOVA ($P<.05$)
	Control	CR	Control	CR	
apoA-I/HDL cholesterol ratio (AU/mmol×10^{-12})	217±36	117±24	406±46	155±7	CR
apoJ/HDL cholesterol ratio (AU/mmol×10^{-12})	500±59	237±19	390±27	198±22	CR
PON1/HDL cholesterol ratio (AU/mmol×10^{-12})	186±24	83±15	180±3	105±8	CR

Values are expressed as the mean±S.E.M. of six animals per group.

effects of CR were observed. apoA-I levels positively correlated with both PON1 activity and levels ($R=.537$, $P=.018$ and $R=.684$, $P=.002$, respectively). Moreover, a significant correlation between apoJ and PON1 serum levels was also found ($R=.602$, $P=.006$).

4. Discussion

Short-term CR (14 weeks of 40% energy restriction) decreases serum PON1 activity in both genders, which is a consequence of a lower number of PON1 molecules in the blood. In fact, the strong correlations between serum PON1 levels and serum activities further support this idea. However, the decrease of PON1 activities of CR animals cannot be attributed to a lower liver PON1 expression, the main source of serum PON1 [3], at least at the transcriptional level.

apoA-I, the main component of HDL, is involved in the stability and function of PON1 [12,13], and its decrease in response to CR in female rats — which has already been described in women [30,31] — would account for the lower serum PON1 levels. In fact, the positive correlations between apoA-I levels and both PON1 activity and levels found in our study are in agreement with results from both in vitro experiments — in which PON1-specific activity was enhanced in the presence of apoA-I [12,13,32] — as well as human apoA-I deficiency states — in which PON1 activity and/or concentration was lower [33]. Thus, a lower stability of the PON1–HDL association under CR can be considered, at least in female rats.

apoJ is mainly present in the HDL_3 subfraction [34], which has been reported to show both the highest PON1 activity [35] and the most effective inhibitory capacity of LDL oxidation [36,37]. Therefore, and in accordance with the strong correlation found between PON1 and apoJ serum levels, the decreased apoJ levels observed in female rats in response to CR further support the idea of a CR-associated decrease in PON1 activities as a consequence of a lower content of HDL particles containing the enzyme. Thus, and in agreement with previous studies in humans [30], we hypothesize that the lower PON1 activity and PON1, apoA-I and apoJ levels of CR groups could be due to a lower serum concentration of the HDL_3 particles, at least in female rats.

The slightly higher paraoxonase and arylesterase activities in the serum of female control rats compared with male rats are in agreement with a previous study performed in mice [38]. Since apoA-I and apoJ serum levels are also higher in female rats, the aforementioned higher PON1 activity could again be attributed to a greater stability of the HDL–PON1 complexes and/or a higher serum content of the PON1 vehicle in female rats. Although the apoA-I and apoJ levels only decreased in CR female rats, the apoA-I/HDL cholesterol and apoJ/HDL cholesterol ratios (Table 3) reveal that, in both genders, CR is associated with a decrease in the contents of apoA-I and apoJ in HDL, which may be responsible for the loss of serum PON1 activity. This fact is confirmed by the PON1/HDL cholesterol ratio (Table 3), which shows that the HDLs of CR rats have a lower content of PON1 molecules than those of control animals. Moreover, the gender differences observed in response to CR in apolipoprotein levels could account for the more marked decrease in PON1 activities shown by female rats compared with male rats.

Liver paraoxonase activity, unlike serum activity, increased in response to CR, which could be indicative of a higher PON1 production by the liver. In spite of the strong correlation between liver PON1 mRNA levels and liver arylesterase activity, the lack of differences in PON1 mRNA levels, liver arylesterase activity and total microsomal protein concentration enables us to suggest that the increase in liver paraoxonase activity in response to CR is not due to modifications at the transcriptional level. Liver PON1 is located in the microsomal fraction, the main site for the catabolism of xenobiotic compounds, and is mainly involved in detoxification mechanisms [39]. Thus, the different responses that serum and liver PON1 show to CR could be explained by the different functions that they have.

Although the decrease in serum PON1 activity in response to CR could be indicative of a loss of antioxidant protection, we propose that it could be part of a strategy leading to energy saving in a situation of decreased nutrient intake and lower risk of oxidative stress in which the protection of LDL particles would be less necessary. This is particularly obvious in females, who promote their own survival and that of the species through mechanisms of energy conservation such as a reduction in heat production [40]. Thus, female rats, which show a higher thermogenic activity than male rats when fed ad libitum, in response to CR undergo a decrease in heat production until they reach male levels [40].

In summary, the PON1 response to CR shows gender-associated differences that are indicative of the different adaptive strategies of male and female rats when faced with a period of food restriction. Thus, in ad libitum feeding conditions, female rats seem to have a more advantageous

status to face a situation of oxidative stress than male rats. Short-term CR attenuates these differences, bringing the response capacity of both genders closer. Since PON1 has been related to the incidence of cardiovascular disease in humans [4], our results could be of interest in the study of the molecular basis of the gender-associated differences in the incidence of these pathologies [41]. To our knowledge, this is the first study demonstrating that CR reduces serum PON1 activity in male and female rats, which could be due to a reduced stability of the HDL–PON1 association and/or the number of HDL particles responsible for PON1 transport and function in the blood.

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