Clinical Endocrinology (2007) **67**, 687–692 doi: 10.1111/j.1365-2265.2007.02946.x

ORIGINAL ARTICLE

The effect of diet on Paraoxonase 1/Arylesterase activities in patients with disorders of galactose metabolism

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Summary

Objective To investigate the effects of diet on the antiatherogenic enzyme Paraoxonase 1/Arylesterase (PON1/Aryl) activities in patients with disorders of galactose metabolism.

Patients and methods Eleven poorly dietary controlled children with classical galactosaemia (GALT deficiency), 7 with epimerase deficiency and 12 with duarte 1 variant 'off diet' underwent clinical and laboratory investigations before and after 10 days on galactose restricted diet whereas controls ($N = 20$) were examined once. Serum lipids, lipoproteins and apolipoprotein A1 (ApoA1) were measured with routine methods, PON1/Aryl activities and total antioxidant status (TAS) spectrophotometrically, and galactose-1-phosphate (Gal-1-P) enzymatically.

Results Lipids, lipoproteins, ApoA1, PON1/Aryl, TAS remained unaltered in all groups, except in those with classical galactosaemia pre- versus postdiet. In patients with classical galactosaemia, TAS, PON1, Aryl (0·98 ± 0·2 mmol/l, 60 ± 12 U/min/ml, 56 ± 16 KU/min/ml, respectively) were significantly reduced prediet as compared with those postdiet (1·63 ± 0·2 mmol/l, 136 ± 15 U/min/ml, 112 ± 18 KU/min/ml, respectively; $P < 0·001$) and controls. The enzyme activities positively correlated with TAS ($r = 0·56$, $P < 0·001$) in all groups and negatively with Gal-1-P ($r = –0·54$, $P < 0·001$) in group with GALT deficiency.

Conclusions Low TAS and high Gal-1-P levels may reduce PON1/Aryl activities. Patients with classical galactosaemia, when on strict diet, may benefit with a generous antiatherogenic capacity.

(Received 9 February 2007; returned for revision 30 March 2007; finally revised 24 April 2007; accepted 4 May 2007)

Introduction

Three inherited disorders of galactose (Gal) metabolism resulting in galactosaemia have been described.[1] The diagnosis is conducted by the detection of Gal and galactose-1-phosphate (Gal-1-P) in blood and is established by the evaluation of the responsible enzymes in peripheral blood cells.[1,2] The most serious variant of the disease is classical galactosaemia due to deficiency of the enzyme galactose-1-phosphate uridyl transferase (GALT deficiency) usually measured in the erythrocytes, as well as complete epimerase deficiency, which resembles classical galactosaemia or partial GALT deficiency as in Duarte variant, which is usually benign.[1] Classical galactosaemia is associated with remarkably high levels of Gal-1-P and galactitol (Galtol) in the tissues. Acute symptoms, such as seizures, lethargy or glucose disturbance and/or chronic jaundice, weight loss, mental retardation, liver and renal dysfunction are implicated with high Gal-1-P concentration in blood, whereas Galtol is associated with cataract.[1,2] Galactose-restricted diet is the only treatment of the disease.[2]

Our previous study[3] showed low total cholesterol (t-chol), low density lipoprotein cholesterol (LDL-C) and apolipoprotein B in patients with various galactose metabolization disorders who were on soybean diet during infancy, whereas high density lipoprotein cholesterol (HDL-C) and apolipoprotein A1 (ApoA1) levels remained unaltered.

In addition, previous *in vitro* study[4] showed that the activity of rat brain acetylcholinesterase (AChE) was modulated after incubation with a mixture containing Gal, Gal-1-P and Galtol concentrations usually found in the blood of patients with classical galactosaemia. Moreover, Gal-1-P was found to inhibit (–70%) pure human AChE.[5] Similarly, in a very recent study, we found the activity of the erythrocyte AChE to be decreased in poorly controlled patients with classical galactosaemia.[6] The enzyme activity was restored nearly to normal when the patients were put on their special diet. In addition, in the same group of patients, we determined a very low antioxidant status (TAS) which was restored when they followed a restriction of Gal intake.[7]

Furthermore, Paraoxonase 1 (PON1)/Arylesterase (Aryl), associated with HDL, were initially identified for their abilities to hydrolyse organophosphate compounds and aromatic carboxylic esters.

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Similarly, AChE, another esterase, is also associated with the action of organophosphorus esters.[5] Recent interest in the enzymes has arisen from the idea that PON1 protects LDL and HDL from the lipid peroxidation.[8] This protection was proposed to be related to the peroxidase-like activity of PON1 on preexisting peroxides and the ability of PON1 to modify the proportion of oxidation products in oxidized LDL[9–11] followed by increased risk of atherosclerosis. In studies with PON1 knockout mice, PON1 was shown to be necessary for the *in vitro* protective effects of HDL on LDL oxidation.[12,13] Recent clinical investigations indicate that PON1 activity is lower in subjects with coronary heart diseases than control subjects.[8,9] Despite the association of PON1 activity with the prevention against LDL oxidation, the mechanism by which PON1 inhibits the oxidation of LDL phospholipids is not clear. Hydroxygen peroxide at millimolar concentrations was observed to partially inactivate PON1·[14,15] Additionally, under oxidative stress conditions, HDL constitute a target for oxidative modifications that may affect their antioxidant properties.[16] It should also be noted that PON1 activity is strongly dependent on its stability, which is enhanced in a phospholipids environment and in association with ApoA1. Nevertheless, there have been few attempts to define the *in vivo* conditions for oxidative inactivation of PON1 and the relationship between oxidative inactivation of PON1 and its antioxidant capacity.[17,18]

As Gal restriction diet is a lifelong diet for galactosaemic patients, especially those with classical galactosaemia, and PON1/Aryl activities are closely related to oxidation and atherogenesis, we aimed to measure their enzyme activities in patients with disorders of galactose metabolism.

Subjects and methods

The present study was approved by the Greek Ethics Committee and was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the parents of the participants.

Subjects

Patients and controls underwent standard laboratory tests (blood count, C-reactive protein (CRP), urine specimen, liver function tests, quantification of amino acids in the sera, or H_3 levels) to exclude any subclinical infection processes before enrolling into the study. The study population consisted of 11 patients aged 8·9 ± 1·2 years (mean ± SD) with classical galactosaemia (Group A), epimerase deficiency (Group E, $N = 7$) and duarte 1 variant (Group D, $N = 12$), who did not adhere strictly to their therapeutic diet as evidenced by the high Gal + Gal-1-P levels in their blood. The patients were requested to follow their special therapeutic diet strictly for 10 days (Group A1, Group E1, Group D1) and then they were reevaluated. They avoided not only food containing galactose/lactose, but also food containing galactosides (e.g. peas, beans, spinach, etc.) or high nucleoprotein (e.g. egg, liver, etc.).[19] Twenty healthy children of comparable age (9·2 ± 1·5 years) were the controls (Group C). All galactosaemic patients were admitted to the day clinic of the Inborn Errors of Metabolism Department (Institute of Child Health) in Athens.

Methods

After 7–8 h fasting venous blood (5·0 ml) samples were collected in blood collection tubes from galactosaemic patients before (Group A, Group E, Group D) and after following their diet strictly (Group A1, Group E1, GroupD1); controls (Group C) were tested once.

Microassay for estimation of blood Gal + Gal-1-P in Guthrie cards

Quantitative analysis of Gal + Gal-1-P was evaluated using a commercial kit (Gal MMR 2000, R & D Diagnostics Co, Athens, Greece). Sensitivity of the assay was 0·6 mg/dl.

The R & D Diagnostics total galactose kit uses trichloroacetic acid (TCA) to extract total galactose (D + galactose and galactose-1-phosphate) from dried blood-spot samples. After extraction, the eluted sample is combined with an enzyme–coenzyme solution containing galactose dehydrogenase (EC 1·1·48), alkaline phosphatase (EC 3·1·3·1) and nicotinamide adenine dinucleotide (NAD). Elimination of the alkaline phosphatase from the enzyme–coenzyme solution will not convert Gal-1-P to D + galactose. Therefore, only D + galactose will be measured. The concentration of Gal-1-P can be then calculated from the difference between the concentrations of total galactose (t-Gal) and D + galactose. Intra- and interassay variations were 4·2% and 3·5%, respectively.[20]

Determination of TAS

TAS was measured in plasma as previously reported by Millet *et al.*[21] Plasma was frozen for up to 14 days before analysis. 2,2′azino-di-3-ethylbenzthiazoline sulphonate (ABTS) was incubated with peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{\bullet+}$, which was a relatively stable, blue-green colour measured spectrophotometrically at 600 nm. Antioxidants in the added sample cause suppression of the above colour production to a degree proportional to their concentration. The assay range was 0–2·5 mmol/l. Samples with concentrations > 2·5 mmol/l were diluted with 0·9% NaCl and reassayed. According to Strube *et al.*[22] the present method calculates both the radical scavenging effect and the effect on the rate of ABTS oxidation. A possible over-estimation of TAS calculation is attributed to all groups studied. Intra- and interassay variations were 3·4% and 3·9%, respectively.

Evaluation of lipids, lipoproteins and ApoA1

Serum t-chol, triacylglycerol, HDL-C, LDL-C, very low density lipoprotein cholesterol (VLDL-C) were measured using ADVIA-1650 Chemistry System (Bayer Corporation, Tarrytown, NY), while ApoA1 serum levels were determined by latex-particle-enhanced nephelometer (Dade Boehring, Liederbach, Germany). Quality control has been previously indicated. Interassay variation coefficients for t-chol, triacylglycerol, HDL-C and ApoA1 were 3·5%, 3·7%, 5·1% and 4·5%, respectively.[23]

Table 1. Serum lipids, lipoproteins and apolipoprotein A1 (ApoA1) in galactosaemic patients pre- and postdiet *vs.* controls

	t-chol (mmol/l)	Triacyl (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	ApoA1 (g/l)
Group A	4·30 ± 0·40	1·15 ± 0·30	1·15 ± 0·16	2·4 ± 0·5	1·55 ± 0·20
A1	4·20 ± 0·40	1·19 ± 0·20	1·16 ± 0·14	2·5 ± 0·5	1·50 ± 0·30
Group E	4·10 ± 0·30	1·13 ± 0·20	1·16 ± 0·15	2·3 ± 0·5	1·52 ± 0·30
E1	4·12 ± 0·30	1·12 ± 0·20	1·18 ± 0·16	2·5 ± 0·4	1·55 ± 0·40
Group D	4·15 ± 0·30	1·14 ± 0·30	1·14 ± 0·20	2·4 ± 0·4	1·60 ± 0·30
D1	4·12 ± 0·40	1·13 ± 0·30	1·14 ± 0·20	2·6 ± 0·2	1·58 ± 0·30
Controls	4·19 ± 0·50	1·10 ± 0·40	1·20 ± 0·18	2·4 ± 0·5	1·70 ± 0·40

t-chol, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; triacyl, triacylglycerol.

Determination of Paraoxonase 1 (PON1)/ Arylesterase (Aryl)

PON1 activity towards paraoxon (O,O-diethyl-O-*p*-nitro-phenyl phosphate from Sigma-Aldrich Co, St Louis, MO) was measured after the reaction of paraoxon hydrolysis into *p*-nitrophenol and diethylphosphate as described previously with modifications. PON1 assays were performed in the absence of sodium chloride (NaCl) (baseline activity) in a buffer containing 90 mM Tris-HCl, pH = 8·5, 1·5 mM $CaCl_2$ and 2·5 mM paraoxon. We measured the rate of hydrolysis of paraoxon at 37 °C by monitoring the increase of absorbance at 410 nm. Frozen aliquots of a serum pool were used as an internal control. At least one aliquot of the serum pool was measured in every 10 samples in order to correct for interassay variations. The enzyme activity was calculated from the molar extinction coefficient of *p*-nitrophenol at 410 nm (18 300 M/cm). IU/ml of paraoxonase was defined as 1 nmol of *p*-nitrophenol formed per min. The intra- and interassay coefficients of variation were 1·89% and 1·37%, respectively.

PON1 activity was also assessed by use of phenylacetate (Fluka, Buchs, Switzerland) as substrate. Aryl activity of PON1 was determined spectrophotometrically in an assay buffer containing 1 mM phenylacetate, 20 mM Tris-HCl (pH = 8·0) and 1 mM $CaCl_2$. Serum samples were prediluted 1 : 8. Blanks without enzyme were used to correct for spontaneous hydrolysis. Frozen aliquots of a serum pool, used as an internal control, were thawed just before the beginning of the assay. One sample of serum pool was measured every 10 samples. Activity was calculated from the molar extinction coefficient at 270 nm of 1310 M/cm on a UNICAM UV2 VIS/UV spectrophotometer. 1 U/ml of aryl activity was defined as a micromole of phenylacetase hydrolysed per min.[24,25]

Statistical analysis

Data were expressed as mean ± SD. Because data distribution was Gaussian, paired *t*-test was utilized for the statistical analyses of the results of the same patients pre- *vs.* postdietary treatment and *t*-test for the comparison of data values between patients and controls. Pearson's test was used for the estimation of coefficient variations. All statistical analyses were performed with an IBM computer using SPSS 10·0 statistical package (SPSS Inc., Chicago, IL). *P*-values < 0·05 were considered statistically significant.

Results

The limitation of this study is the small number of participants per group.

As shown in Table 1, no statistically significant differences were found in lipid, lipoprotein and ApoA1 serum levels not only between the groups of patients but also between patients and controls.

As presented in Table 2, Gal levels were significantly lower in all groups of patients postdiet. Additionally, Gal-1-P levels were significantly lower in patients with classical galactosaemia postdiet. TAS levels and the activities of the enzymes, PON1 and Aryl, were remarkably decreased in Group A than those of Group A1 (pre- *vs.* postdiet) and the other groups of patients and controls. TAS levels as well as the enzyme activities were found unaltered in the other groups of patients pre- versus postdietary treatment and between the patients and controls.

Moreover, positive significant coefficient correlations were found between the enzyme activities with HDL-C ($r = 0{\cdot}74$, $P < 0{\cdot}001$) and ApoA1 ($r = 0{\cdot}64$, $P < 0{\cdot}001$) levels in all the studied groups except those of Group A. In contrast, Gal-1-P levels negatively correlated with PON1 and Aryl activities in the patients with classical galactosaemia before and at the end of their dietary treatment (Table 3), whereas the enzymes positively correlated to TAS in all study groups ($r = 0{\cdot}58$, $P < 0{\cdot}001$). Additionally, Gal-1-P negatively correlated to TAS ($r = -0{\cdot}54$, $P < 0{\cdot}001$).

Table 2. Total galactose (Gal), galactose-1-phosphate (Gal-1-P) blood concentrations, total antioxidant status (TAS) in plasma and the activities of the enzymes Paraoxonase 1 (PON1), Arylesterase (Aryl) in the sera of patients before and after on diet *vs.* controls

	t-Gal (μM)	Gal-1-P (μM)	TAS (mmol/l)	PON1 (U/min/ml)	Aryl (KU/min/ml)
Group A	183 ± 33[a]	285 ± 23[b]	0·98 ± 0·20[c]	60 ± 12[d]	56 ± 16[e]
Group A1	117 ± 28[f]	183 ± 25[g]	1·63 ± 0·20[h]	136 ± 15[i]	112 ± 18[j]
Group E	126 ± 30	16·0 ± 4·0	1·50 ± 0·30	152 ± 14	114 ± 16
Group E1	68 ± 10	12·0 ± 2·5	1·68 ± 0·20	156 ± 15	110 ± 14
Group D	80 ± 8	15·0 ± 3·0	1·75 ± 0·30	139 ± 15	109 ± 15
Group D1	45 ± 6	12·0 ± 3·0	1·80 ± 0·20	146 ± 16	112 ± 14
Group C	55 ± 8[k]	10·0 ± 3·5[l]	1·88 ± 0·30[m]	156 ± 23[n]	109 ± 21°

Statistics: a/f, b/g, c/h, d/i, e/j, f/k, g/l, i/n, j/o = $P < 0{\cdot}001$; a/k, b/l, c/m, d/n, e/o = $P < 0{\cdot}001$.

Table 3. Correlation coefficients between the enzyme paraoxonase 1 (PON1), arylesterase (Aryl) activities and total antioxidant status (TAS), lipid, lipoproteins, apolipoprotein A1 (ApoA1), galactose (Gal) and galactose-1-phosphate (Gal-1-P) in patients with classical galactosaemia pre- (Group A) *vs.* postdiet (Group A1)

	Groups	PON1	Aryl
Triacyl	A	0·14	0·16
	A1	0·13	0·14
t-chol	A	0·12	0·12
	A1	0·13	0·13
HDL-C	A	0·14	0·19
	A1	0·68*	0·54*
LDL-C	A	0·16	0·12
	A1	0·12	0·12
Apo	A	0·16	0·16
	A1	0·66*	0·54*
TAS	A	0·56*	0·56*
	A1	0·60*	0·54*
Gal-1-P	A	–0·54*	–0·56*
	A1	–0·64*	–0·60*
Gal	A	0·12	0·06
	A1	0·13	0·14

Triacyl, triacylglycerol; t-chol, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.
*$P < 0{\cdot}001$.

Discussion

Recently, it was suggested that the enchanced inactivation of HDL-bound PON1 in glycoxidative condition was ascribed to free radicals-induced oxidation. Moreover, α, β-unsaturated aldehydes stable decomposition product of lipid peroxides, which gradually accumulate in the later stage of LDL oxidation, could also cause the inactivation of PON1 by modifying the cysteine residue of PON1 in the same way as it had been observed with *p*-hydroxymercuryl-benzoate (PHMB), a cysteine modifier.[14,26] Thus PON1 is speculated to be one of the antioxidant enzymes very susceptible to oxidate stress in *in vivo* system.

Furthermore, plasma TAS in galactosaemic patients was found to be significantly decreased in those with GALT deficiency on poor diet, whereas no reduction was observed in the patients or the other groups of study prediet. This finding could be explained by the high Gal-1-P levels in their blood: The enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two pentose-cycle enzymes catalysing the production of NADPH from nicotinamide adenine dinucleotide phosphate (NADP), were found to be inhibited by Gal-1-P.[27] NADPH is a significant factor for glutathione reductase, the major enzyme responsible for the regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG),[28] and the production of endothelial NO.[29] Thus, the evaluated high Gal-1-P blood levels in the patients with classical galactosaemia on poor diet may result in a low TAS. Moreover, we cannot exclude the role of minerals in enzyme function and oxidation. Magnesium is a mineral which is a cofactor of the above mentioned enzymes of the pentose cycle.[30] By damaging membrane proteins by free radicals, the activities of important enzymes such as glucose-6-phosphatase and Na^+, K ± ATPase were found to be inhibited.[31,32] As found in a previous study *in vitro*, Mg^{2+} pump was modulated by high Gal-1-P.[27] *In vivo*, Mg^2 ± ATPase activity in the erythrocyte membrane from poorly dietary controlled patients with classical galactosaemia was also found to be significantly decreased, which was partially restored when incubated with reduced glutathione or l-cysteine.[33] It is clear that Mg^{2+} could also play a role in the low TAS levels, affecting the function of the enzymes of the pentose cycle and resulting in free radical production. Obviously, in the patients with classical galactosaemia on strict diet (Group A1) the Gal-1-P levels were found to be higher than those in controls as well as the other groups of patients with galactosaemia, probably due to the endogenous production of the ester[1] which resulted in a higher TAS than that prediet.

In addition, in the last three decades, biomedical literature has implicated free radicals in many pathological conditions. The harmful effects of free radicals lead to oxidative stress of the cells if not neutralized by antioxidants.[34,35]

Plasma lipoprotein abnormalities may even be essential for the common occurrence of atherosclerotic vascular diseases. The abnormalities include elevated concentrations of LDLs and VLDLs and reduced concentrations of HDLs, as generally estimated from measurements of plasma cholesterol, triacylglycerol and HDL-C. Other studies, however, indicate that the most pronounced lipoprotein abnormalities in patients with early onset coronary heart disease are high triacylglycerol and low HDL-C with lesser elevations of LDL-C. In these individuals, the LDL particles are somewhat smaller and denser than in those with a more favourable lipoprotein profile.[35] In this study, lipids, lipoprotein and ApoA1 levels not only did not differ among the studied groups but also pre *vs.* postdiet. Furthermore, PON1/Aryl activities strongly correlated with HDL-C and ApoA1 in patients on strict diet with classical galactosaemia but not with those in the other groups of patients and controls. Also, the antiatherogenic enzyme activities positively and significantly correlated to TAS in all the groups of this study and negatively to Gal-1-P levels (Table 3).

To determine whether the above mentioned associations may be attributed to related alterations in PON1/Aryl activities, or whether the alterations of the enzyme activities are causally related to their long-term exposure to oxidative conditions and high Gal-1-P levels as found in the patients with GALT deficiency, we have postulated three possible explanations: One is that serum PON1/Aryl activities are lowered as a result of an altered synthesis or secretion of HDL-C. These alterations may result from damaged liver cells that are not able to express PON1/Aryl activities as found in rats with experimental cirrhosis.[36] In this study HDL-C, ApoA1 and liver enzyme function tests were found normal in all the participants. Taken together, our data indicate that PON1/Aryl activities may be reduced by their long-term exposure to high hydroxyl radicals' levels, here presented as low TAS.[37] In other words, the enzymes, present in relatively high activities in the physiological system, might scavenge these reactive compounds for a long time at least according to stoichiometric removal mechanisms. Therefore, it is likely that hydroxyl radicals may be active and responsible for the oxidative reduction of the PON1/Aryl in *in vivo* system. This suggestion is further reinforced not only by the restoration of the enzyme activities when the

patients with classical galactosaemia adhere to their therapeutic antioxidant diet strictly and their TAS levels were increased, but also as by the normal PON1/Aryl activities determined in patients with other galactose disorders in whom TAS levels were also high.

Finally, we cannot exclude the possibility that high Gal-1-P levels might lower the enzyme activities as found in our previous *in vitro*[4,5] and *in vivo* studies[6] on another esterase: high Gal-1-P levels decreased both AChE activity in rat brain as well as the erythrocyte membrane AChE activity in patients with classical galactosaemia 'off diet'[6] and pure human AChE.[5] We suggest that both low TAS indirectly and high serum Gal-1-P levels may reduce directly PON1/aryl activities in poorly controlled galactosaemic patients. In addition, PON1 was suggested to protect against cardiovascular diseases secondary to its ability to break down oxidase lipids and to inhibit LDL oxidation.[15] It was reported[38] that this effect was associated with AChE mediated hydrolysis of lipid peroxides which accounts for the inhibition of the onset of LDL oxidation, the oxidative propagation phase and aldehyde formation. As AChE, similar to PON1, can hydrolyse lipid peroxides and prevent the accumulation of LDL, inhibition of these enzymes by free radicals and/or high Gal-1-P levels may lead to early atherosclerosis development.

Conclusions

Remarkably reduced TAS levels were found in poorly controlled patients with classical galactosaemia probably due to the increased Gal-1-P blood levels, whereas the antioxidant capacity was found normal in the other groups of patients versus controls. PON1/Aryl activities were positively correlated to TAS and negatively correlated to Gal-1-P levels. Patients with classical galactosaemia on strict diet may benefit from almost normal PON1/Aryl activities and consequently a normal antioxidant capacity towards LDL oxidation during their life.

Finally, these *in vivo* findings prove once again the significant role of a strict therapeutic diet in the regulation of two antioxidant enzyme activities in patients with classical galactosaemia.

Acknowledgements

The authors are highly indebted to Mrs J. Chadjidakis, D. Tzianou and A. Tsiga for their technical assistance, Mrs Anna Stamatis for her careful typing of this manuscript, and medical student Alexios Mentis for his significant assistance.

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