DOI: http://dx.doi.org/10.48305/arya.v18i0.2319 Published by Vesnu Publications

The effect of homocysteine thiolactone on paraoxonase and aryl esterase activity of human serum purified paraoxonase 1 in vitro experiments

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Original Article

Abstract

BACKGROUND: The important role of lipoproteins, particularly low-density lipoprotein (LDL) and high-density lipoprotein (HDL), has been highly regarded among the known causes of cardiovascular disease (CVD). A wide range of risk factors may cause structural and functional changes in lipoprotein particles, resulting in deposition and formation of atherosclerotic plaques. Homocysteine is one of the most important risk factors in heart disease, and its atherosclerotic properties appear to be related to its intermediate metabolite called homocysteine thiolactone (HCTL). The major aim of the present investigation was to study the effect of HCTL in different concentrations (10, 50, and 100 µM) on paraoxonase and aryl esterase activities of purified human serum paraoxonase 1 (PON1) antioxidant enzyme related to HDL, as an extracellular hydrolyzing enzyme of HCTL.

METHODS: In order to purify PON1 enzyme from human serum, three-step chromatographic methods including DEAE Sephadex A50, Sephadex G100, and DEAE Sephadex A50 were used. Protein concentration and paraoxonase and aryl esterase activities of each fraction were measured separately and the highest activities fractions were collected and subsequently pooled together for the next steps. Ultimately, both activities of PON1 in the presence of different concentrations of HCTL were measured in triplicate by spectrophotometry technique.

RESULTS: HCTL at concentrations of 50 and 100 µM decreased both paraoxonase and aryl esterase activities ($P < 0.05$) in comparison with the control group, which is directly related to the increase in HCTL concentration. However, at a concentration of 10 µM HCTL, no significant difference was observed in both paraoxonase and aryl esterase activities compared to the control group.

CONCLUSION: HCTL is a highly toxic and reactive compound that is produced in all cells. Extracellular enzyme PON1 causes its hydrolysis with high efficiency. The results obtained from the present study showed that paraoxonase and aryl esterase activities decreased in vitro in the presence of HCTL and therefore, HCTL may cause changing in the protein structure of this enzyme. Previous in vivo studies have also shown decrease of PON1 activity in patients with hyperhomocysteinemia.

Keywords: Atherosclerosis; Homocysteine Thiolactone; Paraoxonase 1

Date of submission: 03 Dec. 2020, *Date of acceptance:* 19 July 2021

Introduction

According to studies conducted in recent years, cardiovascular diseases (CVDs) are known as the most important causes of death all around the world. Extensive causes of this disease might be genetic factors, smoking, environmental pollution, and also underlying diseases such as diabetes, hypertension (HTN), hyperlipidemia, etc. All of the mentioned factors may lead to the increase in the incidence of atherosclerotic disorders.[1]

Elevation of plasma lipids following functional

How to cite this article: Moshtaghie E, Nayeri H, Moshtaghie AA, Asgary S. **The effect of homocysteine thiolactone on paraoxonase and aryl esterase activity of human serum purified paraoxonase 1 in vitro experiments.** ARYA Atheroscler 2022; 18: 2319.

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and structural dysfunctions due to the oxidation of important blood lipoproteins including low-density lipoprotein (LDL) and high-density lipoprotein (HDL) by known risk factors can lead to the formation of atherosclerotic plaques. HDL particles are also able to prevent the oxidation of LDL particles through various mechanisms, such as stimulating nitric oxide and prostacyclin producing by endothelial cells, which is able to facilitate better regulation of vascular structure and respiration. The antioxidant properties of HDL are due to its enzymatic content including paraoxonase 1 enzyme (PON1), platelet-activating factor acetyl hydrolase (PAF-AH), glutathione selenoperoxidase, and lecithin cholesterol acyltransferase (LCAT).[2]

In recent years, the association between elevated plasma homocysteine levels as one of the most important atherosclerotic and CVD risk factors has been much considered. Homocysteine is a non-essential sulfur-containing amino acid, which is obtained from the demethylation of methionine by two intermediate compounds: S-adenosyl methionine (SAM) and S-adenosyl-L-homocysteine (SAH).[3]

The most common causes of increased homocysteine are found in people with rare genetic disorders and in drug interactions such as clofibrate, metformin, levodopa, methotrexate, etc.[4] Due to an excessive accumulation of homocysteine, as a result of a genetic error in the pathway of protein biosynthesis, homocysteine is incorrectly selected by transfer ribonucleic acid (tRNA) instead of methionine and a small portion of homocysteine converts to an intermediate metabolite called homocysteine thiolactone (HCTL) via methionyl-tRNA synthetase enzyme (MetRS).[5]

The production of HCTL and the development of hyperhomocysteinemia are known as an independent risk factor in patients with CVD and may cause endothelial cell dysfunction, which is a characteristic of atherosclerosis. As HCTL is formed in all cells and is chemically highly reactive and binds to the ε-amino-lysine group in proteins, which produces toxic homocysteinated proteins and may induce autoimmune responses that eventually lead to atherosclerosis,[6] it is essential to be detoxified by intracellular bleomycin hydrolase (BLH) enzyme and extracellular PON1 enzyme.[7] Due to the low tendency of HCTL to intracellular hydrolysis by BLH in vivo, most of it may be hydrolyzed by lactonase activity of PON1 extracellularly.[8]

The aim of the present in vitro study was to investigate the effect of different concentrations of HCTL on the paraoxonase and aryl esterase activities of human serum purified PON1, as an external hydrolyzing lipoprotein dependent enzyme, due to the lack of accurate information about the mechanism of action of hyperhomocysteinemia as a risk factor of atherosclerosis.

Materials and Methods

Serum preparation: Blood sample was withdrawn from a healthy fasting person (inclusion criteria were non-diabetes and hyperlipidemia) with written consent (IR.IAU.SHK.REC.1397.043). Serum was separated from blood with centrifugation in Isfahan Cardiovascular Research Center, Isfahan, Iran, in 2019 and the levels of biochemical parameters including LDL, HDL, triglycerides (TGs), cholesterol, liver enzymes, and fasting blood sugar (FBS) were measured in serum using an autoanalyzer (Hitachi 902). Triton X-100 was used as a non-ionic detergent at 1% (v/v) to separate PON1 from HDL. The serum treated with triton X-100 was then placed on the mixer at 4° C for 4 hours and after 5 minutes of centrifuge (5000 rpm).

Purification of PON1 by ion exchange chromatography: At first stage, according to Golmanesh et al. method,[9] 2.5 cm x 60 cm column was selected. Column was then loaded with 10 g of DEAE Sephadex A50 resin in buffer A containing 20 mM Tris hydrochloride (Tris-HCL) (pH = 8), 5 µM ethylenediaminetetraacetic acid (EDTA), triton X-100 (0.01% v/v), 1 mM calcium chloride ($CaCl_2$), and 2% v/v glycerol. In order to have equilibration at pH 8, the column was washed with buffer A for 4 hours and the serum sample prepared in the previous steps was loaded on the top of column. Sodium chloride (NaCl) solution in buffer A (0 mM to 700 mM) was prepared to make a concentration gradient and connect to the column by the help of peristaltic pump. 2ml fractions were collected and protein concentration and paraoxonase and aryl esterase activities were measured in each of the 70 fractions.

Purification of PON1 by gel filtration chromatography: 10 g of G100 resin was placed in buffer A at room temperature overnight. The sample prepared from the first chromatographic step was loaded onto the G100 column. The column was then eluted with buffer A. The protein concentration and paraoxonase and aryl esterase activities were measured in all fractions. Those fractions with highest enzyme activities were pooled together and used for third step of chromatography.

Purification of PON1 by ion exchange chromatography: 10 g of DEAE Sephadex A50 resin was dissolved in buffer B containing 20 mM phosphate pH = 7, 5 µM EDTA, 5% glycerol, 0.1% triton X-100, and 1 mM $CaCl_2$ overnight at room temperature. The column was washed with buffer B until the adsorption at 280 nm of the eluted fraction reached to zero approximately. The prepared sample from second step was then loaded onto the column with a concentration gradient of 0 to 600 mM NaCl. Finally, the 70 fractions (2ml) were collected and paraoxonase and aryl esterase activities were then measured by spectrophotometry. The fractions with highest paraoxonase and aryl esterase activities were chosen and pooled together.

Dialysis of purified enzyme samples: Following separation of the enzyme from human serum, dialysis was used to purify and remove substances that might interfere in the assay. A dialysis sac (cut-off of 12000 kDa) was prewashed and the enzyme sample was dialyzed for 24 hours in the presence of buffer B. The dialysate was changed with fresh buffer every 8 hours for three times.

Determination of paraoxonase activity of PON1: The paraoxonase (organophosphate hydrolase) activity of PON1 was measured by Beltowski protocol in which the initial rate of paraoxon substrate hydrolysis to paranitrophenol (p-nitrophenol) was calculated. Buffer solution containing 100 mM of Tris-HCL and 2 mM of $CaCl_2$ with a final concentration of 2 mM paraoxon was prepared (pH = 8) (assay solution). An aliquot of 10 µl prepared enzyme was added to the assay solution (final volume: 520 µl) and p-nitrophenol production rate at 412 nm was determined by spectrophotometric method. Finally, paraoxonase activity was calculated using equations (1) and (2):

$$\text{PON1 Activity (U/l)} = \Delta A/\text{minute} \times F \quad (1)$$

$$F = (\text{total volume µl}/\text{sample volume µl})/(\text{micro molar extinction coefficient}), \text{ where micro molar extinction coefficient} = 0.018290. \quad (2)$$

Determination of aryl esterase activity of PON1: Aryl esterase activity of PON1 was also determined by calculating the initial hydrolysis rate of phenyl acetate substrate. According to the Beltowski protocol, a buffer solution containing 100 mM of Tris-HCL and 2 mM of $CaCl_2$ in the final concentration of phenyl acetate 2 mM was prepared (pH = 8) (assay solution). Production of phenol from hydrolyzing phenyl acetate was determined by adding 10 µl of prepared enzyme to the assay solution (final volume: 520 µl) and absorbance at 270 nm through spectrophotometry. According to equations (1) and (2), aryl esterase activity was calculated by micro molar extinction coefficient = 0.001310.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): In order to ensure the purification process of PON1 enzyme, SDS-PAGE was performed.

Measurement of paraoxonase and aryl esterase activities of PON1 in the presence of HCTL: Enzyme paraoxonase and aryl esterase activities were measured in triplicate in the presence of 10, 50, and 100 µM HCTL concentrations by spectrophotometry technique at 412 and 270 nm, respectively.

Statistical analysis: In this study, data were reported as mean ± standard deviation (SD). In order to analyze the obtained results, SPSS software (version 18, SPSS Inc., Chicago, IL, USA) was used. One-way analysis of variance (ANOVA) with Tukey test as a post-hoc test was applied to compare the effect of different concentrations of HCTL on paraoxonase and aryl esterase activities of PON1. P-value less than 0.05 was considered as statistically significant.

Results

Biochemical parameters: Prior to carry out the experiments, initially the serum parameters related to lipid metabolism were measured by autoanalyzer which are given in table 1.

Table 1. Results of measuring the biochemical parameters of serum samples

Biochemical parameters	Value (mg/dl)
FBS	82
Cholesterol	136
TG	190
HDL	38
LDL	133
SGOT	29
SGPT	55
ALP	138

FBS: Fasting blood sugar; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; SGOT: Aspartate aminotransferase; SGPT: Alanine aminotransferase; ALP: Alkaline phosphatase

Elution of PON1 from chromatography column: As mentioned in "Materials and Methods" section, the serum was loaded on the top of the column and 70 fractions were eluted. The absorbance at 280 nm for each individual fraction was measured (Figure 1).

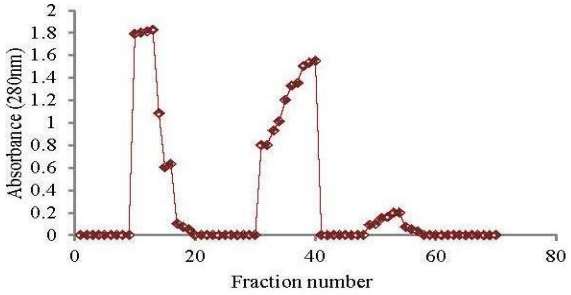

Figure 1. The graphs of absorbance (280 nm) in 70 fractions of DEAE Sephadex A50

The paraoxonase and aryl esterase activities were also measured in 70 fractions and the highest activities were observed in 37-44 fraction. The data from the third column, DEAE Sephadex A50, were given in figure 2.

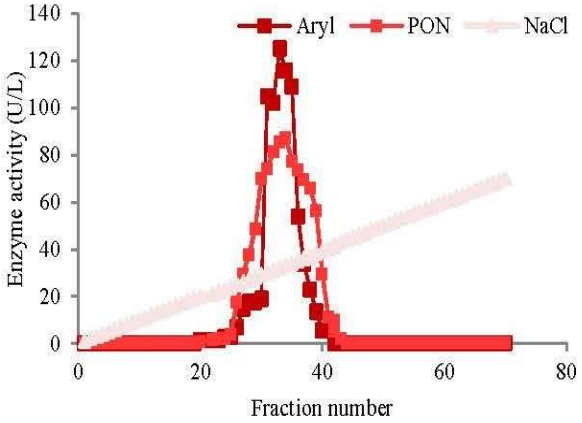

Figure 2. The graphs of paraoxonase (412 nm) and aryl esterase (270 nm) activities in 70 fractions of DEAE Sephadex A50 chromatography

SDS-PAGE of PON1: In order to ensure the purification process, fraction samples (37-44) were pooled from each chromatographic step loaded onto SDS-PAGE and a single band of pure enzyme with a molecular weight of about 43 kDa was shown (Figure 3).

Evaluation of PON1 activities in the presence of HCTL by spectrophotometry: In figures 4 and 5, comparison of mean and SD of PON1 activities, including aryl esterase activity and paraoxonase activity at different concentrations of HCTL (10, 50, and 100 µM), was shown. In both analyses, the effect of HCTL on PON1 activities (paraoxonase activity and aryl esterase activity) was reported significant when P-value was lower than 0.01. The results showed that by increase of HCTL concentrations, both paraoxonase activity and aryl esterase activity were lessened.

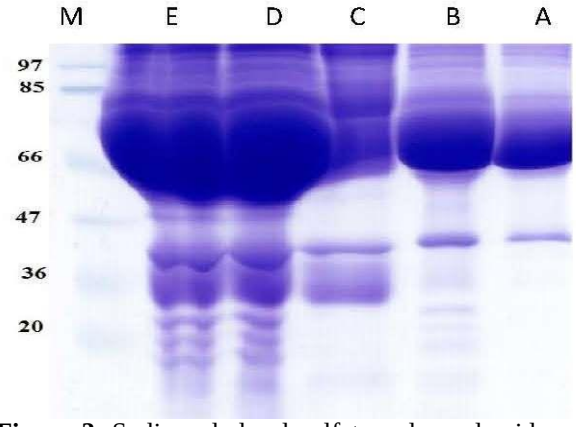

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) bands of purified paraoxonase 1 (PON1) (M: Marker; E: Serum; D: Serum + triton X-100; C: Sample of the highest activity of pooled fractional of first column, Sephadex A50; B: Sample of the highest activity of pooled fractional of second column, G100; A: PON1 of third column, Sephadex A50)

There was no statistical difference in both paraoxonase and aryl esterase activities at a concentration of 10 µM HTCL (P = 0.191, P = 0.792, respectively) when compared with the controls groups.

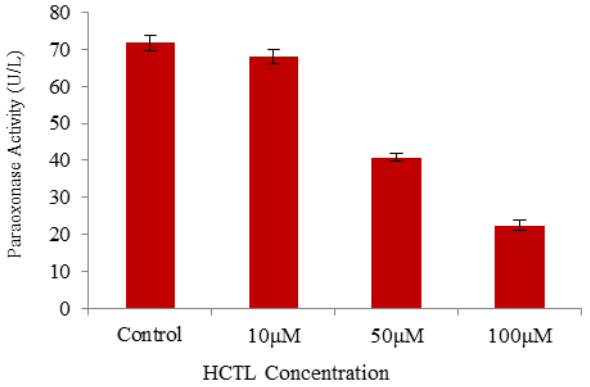

Figure 4. Comparison of paraoxonase activity [mean ± standard deviation (SD)] at different concentrations of homocysteine thiolactone (HCTL). HCTL concentrations of 50 and 100 µM have been shown to cause significant decreases (P = 0.001) in paraoxonase activity in comparison to control group, while lower change in paraoxonase activity was observed in 10 µM of HCTL, which was not statistically significant (P = 0.191)

The most effective concentration of HCTL seems to be related to 100 µM of HTCL, in which the paraoxonase and aryl esterase activities were reduced to 22.5 ± 1.5 and 53.1 ± 2.3, respectively.

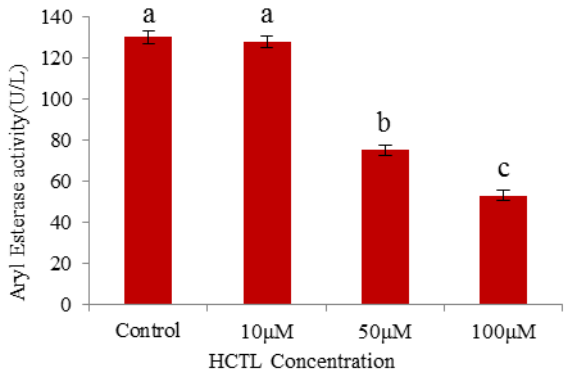

Figure 5. Comparison of aryl esterase activity [mean ± standard deviation (SD)] at different concentrations of homocysteine thiolactone (HCTL); HCTL concentrations of 50 µM and 100 µM have been shown to cause significant decreases (P = 0.001) in aryl esterase activity in comparison to control group, while lower change in aryl esterase activity was observed in 10 µM of HCTL, which was not statistically significant (P = 0.792)

Discussion

Data presented in this study showed that there was a close relationship between hyperhomocysteinemia and low serum level activity of PON1 (Figures 4 and 5). Our findings are consistent with a previous work reported by Yilmaz[10] performing in vitro experiment.

The concept of HDL dysfunction in CVD was introduced in the mid-1990s. The first evidence of proteins replacement such as apolipoprotein A-I (ApoA-I) and paraoxonase was seen during inflammatory responses with acute phase proteins such as ceruloplasmin and serum amyloid A (SAA).[11] Homocysteine reduces plasma HDL cholesterol levels by inhibiting hepatic synthesis of ApoA-I.[12] In fact, homocysteine decreased serum HDL levels by affecting the expression of ApoA-I gene in the liver of laboratory models of treated rats.[13]

Confirming the previous studies, the results of present investigation showed that in concentrations of 50 and 100 µM, HCTL was able to significantly ($P < 0.05$) reduce paraoxonase activity by 43.16% and 68.65% and aryl esterase activity by 42.22% and 59.12%, respectively, in comparison to control groups. However, this decrease in concentration of 10 µM HCTL was not significant in both paraoxonase and aryl esterase activities.

It seems that the relationship between hyperhomocysteinemia and reduction in serum HDL levels in patients can be clearly explained through the results obtained in this in vitro study with the negative effect of different concentrations of HCTL on paraoxonase and aryl esterase activities.[14] In a study aimed at analyzing the activity of PON1 enzyme in patients with endometrial cancer who underwent primary surgery, according to the role of PON1 in the metabolism of HCTL, the amount of HCTL as well as the total serum HCTL concentration was measured. Serum paraoxonase and aryl esterase activities of PON1 with paraoxon and phenyl acetate substrates were significantly lower than the control group. There was also no clear association between Q192R PON1 gene polymorphism and endometrial cancer. Despite the decrease in PON1 activity, no significant difference was observed between the concentration of homocysteine and N-homocysteinylation protein in endometrial cancer with the control group.[15]

Conclusion

In this study, we tried to investigate the effect of HCTL, a homocysteine intermediate metabolite as an important risk factor for heart disease, on paraoxonase and aryl esterase activities of its extracellular hydrolyzing enzyme, PON1. Forasmuch as HCTL is a highly reactive compound, N-homocysteinylation reactions appear to cause structural and functional changes in exposed proteins, which are clearly demonstrated in the present in vitro study. Previous studies have reported in vivo conditions. Therefore, it can be concluded that in patients with hyperhomocysteinemia, the antioxidant activity of HDL related to PON1 is reduced and therefore, following the oxidation and homocysteination of LDL and HDL lipoproteins, the possibility of atherosclerotic plaques formation in the vessel wall increases.

Acknowledgments

This study was supported by Islamic Azad University, Falavarjan Branch, Isfahan (17230528962022), with the cooperation of Isfahan Cardiovascular Research Center. The authors would like to thank all the research laboratory staff of Islamic Azad University, Falavarjan Branch and Isfahan Cardiovascular Research Center.

Conflict of Interests

Authors have no conflict of interests.

Authors' Contribution

AAM and HN conceived the presented idea and developed the theory. HN and SA verified the

analytical methods, encouraged, and supervised the findings of this work. EM carried out the experiment and wrote the manuscript with support from all authors. All authors discussed the results and contributed to the final manuscript.

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