Homocysteinethiolactone and Paraoxonase - novel markers of Diabetic Retinopathy

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Running title: PON-HTLase and diabetic retinopathy

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**Objective:** Paraoxonase (PON), exhibits esterase activity (PON-AREase) and lactonase activity (PON-HCTLase) which prevent LDL oxidation and detoxify Homocysteine thiolactone (HCTL). The role of HCTL and HCTLase as a risk factor for the microvascular complication in diabetic retinopathy at the level of vitreous has not been looked into.

**Method:** Undiluted vitreous from Proliferative diabetic retinopathy (PDR) and Macular hole (MH) (n=13, n=8) were used to determine PON-HCTLase and PON-AREase activity spectrophotometrically. HCTL level was detected by LC-MS/MS. *In vitro* studies were done in primary cultures of bovine retinal capillary endothelial cells (BREC) cells for dose and time dependent effect of HCTL and Homocysteine (Hcys) on the PON-HCTLase activity as well as mRNA expression by RT PCR.

**Results:** A significant increase in HCTL and PON-HCTLase activity was observed in PDR compared to MH (p=0.036, p=0.001), with a significant positive correlation between them (r=0.77, p=0.03). The *in vitro* studies on BREC showed a dose and time dependent increase in the PON-HCTLase activity and mRNA expression of PON2 on exposure to HCTL and Hcys.

**Conclusion:** This is the first report showing elevated levels of vitreous HCTL and PON–HCTLase activity in the PDR, probably a protective effect to eliminate HCTL which mediates endothelial cell dysfunction. Thus vitreous levels of HCTL and PON activities can be markers of diabetic retinopathy. The bioinformatics analysis reveals that the structure - function of PON that can be modulated by hyperhomocysteinemia in PDR can affect the dual enzyme activity of PON.

Hyperhomocysteinemia is a well-established independent risk factor for the development of macrovascular and microvascular diseases (1). Recent reports shows increased HCTL levels associated with diabetic macrovasculopathy (2). HCTL is formed in all cell types when there is excess homocysteine, as a result of error editing met-tRNA synthetase. Interaction of homocysteine thiolactone with proteins leads to protein homocysteinylination and loss of function (3). Therefore detoxification of HCTL is crucial. This is possible by the HCTLase activity of PON. (4). The enzyme PON is a calcium dependent 45 kDa protein, coded by chromosome 7q21-22. The PON gene family in human has 3 members, PON1, PON2 and PON3. While PON1 and PON3 are associated with serum HDL (5), PON2 is ubiquitously expressed in tissues (6). PON1 exhibits antioxidant activities, thereby preventing the accumulation of oxidized LDL and PON2 acts mainly at the cellular level (7). Lipid oxidation plays a role not only in the macrovascular diseases but also in the microvascular dysfunction and serum PON1 activity was decreased in patients with diabetic retinopathy (8). While elevated Hcys in the vitreous of PDR was reported by us and others (9; 10), there are no reports on HCTL levels and PON activity. This study aims at detecting the vitreous levels of HCTL and PON-HCTLase/AREase activity in PDR cases along with *in vitro* studies in BREC.

**RESEARCH DESIGN AND METHODS**

All experiments involving human subjects adhered to the tenets of the Declaration of Helsinki. In patients with PDR, the clinical
Ocular findings were graded at the time of vitrectomy for the presence of hemorrhage, tractional retinal detachment, and presence or absence of patent new vessels in the retina or optic disc. MH patients with, idiopathic full thickness retinal defect of more than 400 µm with posterior vitreous detachment were included as disease control. Clinical details of the patients with PDR and MH are given in Tables (Appendix). Undiluted vitreous samples from 13 patients (mean age, 52 ± 7; 7: M and 6: F) DR and 8 MH (mean age 56 ± 10; 5: M, 3: F) were collected during vitreoretinal surgery, centrifuged and frozen at -80°C. Vitreous HCTL levels, PON-HCTLase activity, total protein, Thiobarbituric acid reacting substances (TBARS), Total antioxidant capacity (TAC) and total thiols were measured.

**In vitro experiments in BREC:** The primary bovine retinal capillary endothelial cells (BREC) were cultured and characterized as endothelial cells using Factor VIII and VE-Cadherin. The cells were exposed to varying concentration (25, 50, 100 and 200 µM) of Hcys and HCTL at varying time points (3, 6, 12, 24 & 48 h) in DMEM/F12 media. The PON-HCTLase and AREase activity were estimated in the cell lysates.

**Homocysteine thiolactone was estimated using LC MS/MS:** The liquid chromatography separation of HCTL in the vitreous was done by gradient elution using acetonitrile with 0.1 formic acid as (A) and water with 0.1% formic acid (B) in the ratio 70:30, pumped at a flow rate of 0.5 ml/min in a Thermo Surveyor quaternary HPLC pump (Thermo Electron Corp, Waltham, MA, USA) coupled with Applied Bio Systems 4000 Q Trap (ABS Biosystems, Foster City CA, USA) and with Positive Electron Spray Ionization mode (ESI). The analytical separation was achieved by using Chromolith, SpeedROD, RP-18e (50 X 4.6 mm) (Merck, Darmstadt, Germany) within the run time of 5 minute where homatropine was used as internal standard. Analyst software version 1.4.2 was used to control all the parameters of mass spectrometry. Quantification was performed using multiple reaction monitoring (MRM) mode based on the parent → product ion transitions for homocysteine thiolactone (118.2→56) and homatropine (276.1→142). Source dependent parameters optimized were gas 1 (40 psi); gas 2 (40 psi); curtain gas (10 psi); ion spray voltage (5500 V) and temperature (300 ℃). Compound dependent parameters declustering potential, entrance potential, collision energy and cell exit potential and dwell time were set at 75, 10, 35, 10 and 200 for both analyte and the internal standard respectively. 20 µl of either standard (100 - 3.125 ng/ml) or sample was mixed with 200 µl of extraction solvent (70:30 ratio of acetonitrile:water with 10% zinc sulphate) containing homatropine at the concentration of 250 ng as an internal standard (11; 12).
Determination of Aryl Esterase activity:
PON-AREase activity was measured using the method of Cabana et al (13). The rate of hydrolysis of substrate PA was measured spectrophotometrically in kinetic mode by detecting the increase in phenol concentration at 270 nm. Undiluted vitreous was added to the buffer consisting of 10 mM Tris and 1mM CaCl₂, pH 8.0. Enzyme activity was expressed as μM PA hydrolysed/ml/min.

Determination of Homocysteine Thiolactonase activity:
HCTLase activity assay was standardized in-house using γ-TBL as the substrate and the rate of hydrolysis was measured spectrophotometrically in kinetic mode at 450 nm (main wavelength) and 546 nm (subwavelength) suitably modifying the method of Koubaa et al. 5 µl of vitreous sample was used for the assay with DTNB as chromogen at pH 7.2 using 100 mM phosphate buffer. Enzyme activity was expressed in U/L.

Activity stain for paraoxonase using phenyl acetate as substrate:
The AREase activity of PON protein in the vitreous was observed by doing an activity stain using PA as substrate. The liberated phenol couples with the hexazotized pararosaniline solution to give an insoluble brightly colored azo dye seen as pink bands in the gel. Briefly 50 µg of the vitreous protein from MH and PDR was run on a native page (12%). The gel was then immersed in a staining solution (Pararosaniline: 0.125 M, Sodium nitrite: 4%, phenylacetate: 1mM in phosphate buffer pH 6.8-7.2.), for 1 hr at 37°C. Once the bands were visualized, the gel was destained with 0.33% sodium meta bisulphate in phosphate buffer. (14).

Determination of Thiobarbituric Reactive Substance (TBARS):
Estimation of vitreous TBARS was done spectrophotometrically based on absorbance of the chromophore at 530nm. The results were expressed as nmol MDA released /ml/mg protein. (15)

Determination of Total Antioxidant Capacity (TAC):
Estimation of vitreous TAC was done spectrophotometrically by a Fenton type reaction. Antioxidants from the added sample of human fluid suppress TBARS production and measured at 532 nm. (16).

Determination of Total Thiols:
Estimation of Total Thiols (a disulphide compound that is readily reduced) was measured spectrophotometrically using DTNB as chromogen at 412 nm. (17).

Statistical analysis:
Students “t” test was used to compare the continuous variables between groups. Pearson’s correlation was employed to calculate the ‘r’ value. Statistical significance was defined as p < 0.05. The statistical analysis was done using SPSS version 14.0.

RESULTS
PON-HCTLase/AREase activity in vitreous:
The PON-HCTLase activity in the vitreous of PDR cases was found to be significantly elevated with a mean of 175.17 ± 16.4 U/L compared to MH (78.5 ± 12.7 U/L) (p=0.00). Correspondingly a significant decrease in PON-AREase activity was observed in PDR (1.5 ± 1.7)μmoles/ml/min cases compared to MH (3.8 ± 1.6 μmoles/ml/min)(p = 0.00). (Table I). Distribution of PON-AREase and PON-HCTLase activity shows a shift in the median with 9 fold drop in the PON-AREase activity and a 2.2 fold increase in PON-HCTLase in the PDR cases when compared to MH. (Figure 1A, B).

There were 6 cases in PDR who were on atorvastatin treatment and there was no significant difference in the activity of PON-HCTLase and PON-AREase in this group. Activity staining for PON AREase revealed the presence of PON protein in the vitreous (Figure 1C).

In order to see if this increase in PON-HCTLase is associated with increase in HCTL levels its level were detected in the vitreous by mass spectrometry. (Figure 1D, E, F). There was a significant
Correlation between HCTLase/AREase with HCTL: A significant positive correlation was observed between vitreous HCTL levels and HCTLase activity, in PDR and MH n=3 (r =0.88, p= 0.03) (Figure 1H). However no significant correlation was observed with PON-AREase and HCTL.

Oxidative stress parameters in the Vitreous: There was a significant decrease in total thiols in PDR cases compared to MH (p= 0.00), with significant increase in the TAC levels (p=0.0001). This increase in TAC value in spite of reduced thiol status can be attributed to the cumulative effect of small molecule antioxidants like Vitamin E and C. Izuta et al suggests that the thioredoxin and Nrf2/ARE pathways can also mediate the redox status in the vitreous body of PDR cases and has reported increased antioxidant potential in the vitreous of PDR (18). However the alteration in the TBARS levels was not significant with the median showing 11.6 in PDR (IQ range: 22.3) and 26.05 in MH (IQ range 2.7). Total protein level in was found to be significantly elevated in PDR (p=0.002) (Table I).

In vitro studies on BREC cells: MTT assay revealed that the cells were viable at all the concentrations of HCTL and Hcys tested until 48 hours . The in vitro experiments showed a dose and time dependent increase in HCTLase activity, when exposed to both HCTL and Hcys (Figure 2A,B). HCTLase activity was found to be significantly increased and maximal at 200 µM at 24 hours for both HCTL & Hcys exposure, compared to untreated control (p=0.000). Correspondingly, the PON-AREase activity was significantly decreased. (p= 0.000) (Figure 2C) In the same experimental condition, the mRNA expression of PON enzyme was tested and an increase in expression was seen for both HCTL and Hcys, the effect was much pronounced in Hcys. (Figure 2D a and b).

DISCUSSION
Homocysteinyllated proteins mediates the development and progression of both diabetic macrovasculopathy and microvascular complications (19) (20). However, its impact on the microvascular endothelial cell is still not well understood. Increased levels of Hcys has been reported to increase the concentration of HCTL levels (3). Serum HCTLase and AREase activity of PON were reported to be significantly lowered in diabetic patients (8). In this study for the first time we observed the vitreous HCTL and HCTLase activity is significantly increased in the vitreous of PDR cases compared to MH. The increase of PON in vitreous could be contributed by the inner retinal barrier permeability and proliferating endothelial cells which are characteristic of PDR. PON2 which is ubiquitously present in all tissues, has the highest lactonase activity, though all the three isoform exhibits it (6). The in vitro study supports this fact wherein exposure of Hcys and HCTL increases the PON2 mRNA expression. Phylogenetic analysis suggests that PON2 is the oldest member of the family. It is reportedly present intra cellularly in 3 major vascular cell types, namely cultured human umbilical vein endothelial cells, smooth muscle vascular cells and aortic adventitial fibroblasts with major function of reducing the reactive oxygen species mediated endothelial cell dysfunction (21). In this study, the BREC cells on exposure to HCTL were found to increase the HCTLase activity. Comparatively this was 2 to 3 fold higher in the Hcys exposure. The mRNA expression of PON2 was increased similar to the activity. Concomitantly there was a significant drop in the AREase activity both in the HCTL and Hcys treated BREC cells. The differential effect seen in Hcys and
HCTL treated may be explained by the
differential uptake of the molecules and
characteristic of cell type. The retinal
capillary endothelial cell uptake of Hcys and
HCTL has not been looked into. However,
Hcys transport is differentially regulated in
vascular cells. In endothelial cells, Hcy
transport is predominantly mediated by a
sodium/lysosome-dependent system ASC
with low transport activity (22). In order to
see if the binding characteristics of the PON
with Hcys alters the HCTLase and AREase
activity, we took a bioinformatics approach.
PON2 bound to two Ca\(^{2+}\) ions was modeled
using MODELER 9V7, (23) based on the
crystal structure of PON1 (1V04) as template
given by Hasel et al.(24) Since the binding of
Hcys to PON2 protein is not known, blind
docking was done Figure 3A, B. The docking
study reveals that Hcys binds to the same
pocket to which HCTL and PA docks. Table
II .Therefore it is quite possible that Hcys can
affect the HCTLase and AREase activity of
PON2. This is the first report showing the
increased activity of PON-HCTLase at the
level of vitreous, may be a protective effect to
eliminate HCTL which mediates endothelial
cell dysfunction through N-
homocysteinylation of the lysine residues in
the proteins (25) , while the PON-AREase is
lowered which is probably due to the
increased Hcys levels. Vitreous levels of
HCTL and PON activities can be markers of
diabetic retinopathy. However the structure-
function that determines the dual enzyme
activity of PON which is altered by molecules
like Hcys, such as in PDR warrants further
attention.

**Author’s contribution:** Barathi. S\(^1\): Researched data, contributed to discussion, wrote manuscript and reviewed/ edited manuscript; Angayarkanni. N\(^1\) : Researched data, contributed to discussion, wrote manuscript and reviewed/ edited manuscript;
Aarthi. P\(^1\) : Researched data;
Sulochana . K. N\(^1\) : Researched data and contributed to discussion; Rishi . P\(^2\) : Researched clinical data, contributed to discussion, wrote manuscript and reviewed/ edited manuscript; Maneesh. D\(^2\) : Researched clinical data; Velpandian. T\(^3\) : Researched Mass spec data; Charanya. M\(^3\) : Researched Bioinformatics data; Muthukumaran. S\(^3\) : Researched Bioinformatics data.

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**Conflict of interest:** No conflict of interest to
disclose.

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Table I: Homocysteine thiolactonase, Aryl esterase activity and oxidative stress parameters in the vitreous of PDR compared to MH cases

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Macular Hole (n = 8)</th>
<th>PDR (n = 13)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine Thiolactonase (U/L)</td>
<td>78.5 ± 12.7</td>
<td>175.1 ± 16.4</td>
<td>p = 0.000</td>
</tr>
<tr>
<td>Arylesterase activity (µmoles/ml/min)</td>
<td>13.8 ± 1.6</td>
<td>1.5 ± 1.7</td>
<td>p = 0.000</td>
</tr>
<tr>
<td>TBARS (nmol/ml/mg protein)</td>
<td>24.42 ± 5.2</td>
<td>17.1 ± 15.2</td>
<td>Median 26.05 (IQ range: 2.7)</td>
</tr>
<tr>
<td>TAC (mmol)</td>
<td>0.22 ± 0.03</td>
<td>0.319 ± 0.24</td>
<td>Median 11.6 (IQ range: 22.3)</td>
</tr>
<tr>
<td>Total Thiols (µmol)</td>
<td>43.88 ± 6.3</td>
<td>28.7 ± 12.9</td>
<td>p = 0.000</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>1.29 ± 0.2</td>
<td>2.83 ± 2.5</td>
<td>p = 0.002</td>
</tr>
</tbody>
</table>
Table II: Binding kinetics of the substrates HCTL and PA and the ligand HCys with PON2 protein.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Binding Energy (kcal/mol)</th>
<th>Residues involved in Hydrogen bonding</th>
<th>Residues involved in hydrophobic interactions</th>
<th>Inhibitory constant (Ki)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMOCYSTEINE</td>
<td>-5.08</td>
<td>Asp168, Ile169, Ile225, Thr170, Ile 55</td>
<td>Ile116, Leu270, Asn226, Asp56, Ca 355</td>
<td>188.88 μM</td>
</tr>
<tr>
<td>HOMOCYSTEINE THIOLACTONE</td>
<td>-6.63</td>
<td>Asp168, Ile169, Ile225, Thr170</td>
<td>Ser117, Ile116, Asn226, Leu270, Ca355</td>
<td>13.87 μM</td>
</tr>
<tr>
<td>PHENYLACETATE</td>
<td>-4.73</td>
<td>Thr118, Ala171</td>
<td>Ser117, Ile225, Ile116, Ile169, Leu270, Thr170, Asn226, Ca 355</td>
<td>338.45 μM</td>
</tr>
</tbody>
</table>
Figure 1: PON activity and the HCTL levels in Vitreous in PDR and MH cases:
Distribution graph showing reciprocal relationship of HCTLase and AREase in PDR (n=13) and MH (n=8). (A) AREase activity, (B) HCTLase activity (C): Activity staining for PON protein in the vitreous using phenyl acetate as substrate and parasoaniline as chromogen. The band was observed at 66 KDa. (Lane 1: MH, Lane 2-4: PDR. Lane 5: HMW marker), Representative LC/-MS/MS chromatogram showing the HCTL (left) and the corresponding internal standard namely Homatropine (right) (D) Standard (E) MH vitreous (F) PDR vitreous. The m/z of HCTL is 118.2 and Homatropine is 276.1 (seen as the peak).(G): Distribution of HCTL levels in PDR (9) and MH (3) cases. Correlation between HCTL and HCTLase (H): PDR (n=9) (Black rhombus), MH (n=3) (Black circle).
**Figure 2: In vitro experiments:**
Figure 2: HCTLase activity in BREC cells exposed to Hcys and HCTL
   A: Graph showing the dose and time dependent increase in HCTLase activity after treatment with HCTL
   B: Graph showing the dose and time dependent increase in HCTLase activity after treatment with Hcys

Figure 2C: AREase and HCTLase activity in BREC cells exposed to Hcys and HCTL at 200 µM compared with the baseline control activity.

Figure 2Da and b: mRNA expression of PON2 in BREC cells exposed to Hcys and HCTL (200µM at 24 h). The PCR was carried out using the following primers for bovine glyceraldehyde 3 phosphate dehydrogenase GAPDH Forward primer 5'-TGTTCAGTATGATTCCACCT-3' and Reverse primer 5'-GTCTTCTGGGTGCGAGTGAT-3' corresponding to 424 bp and for PON2 Forward primer: 5' - CCT TCC TAA TTG CCA CCT GA – 3' and Reverse primer: 5’ – TGG AGG CCT GGA CAT TTT AG – 3’. Corresponding to ~150bp size. The bands obtained were quantified using NIH image J software after normalization to GAPDH.
Figure 3: Bioinformatic analysis of PON2 interaction with Heys

Figure 3A: Shows the residues of the PON2 protein which has hydrogen bonding with the ligand Heys

Figure 3B: Shows the residues of the PON2 protein which has hydrophobic interaction with the ligand Heys