

Mutated LXR- A Gene within Blood Mononuclear Cells of CHD Patients: Significance of Serum Factors

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Abstract

Several lines of evidence attest to the fact that the Liver X Receptor (LXR) - α dependent cross talk between lipid metabolism and inflammation may be of crucial importance in atherogenesis. The present study unambiguously revealed that the serum derived from coronary heart disease (CHD) patients does not possess any factor that had the inherent capacity to induce the observed LXR- α gene mutation in such patients. Surprisingly our experiments clearly indicated that vitamin D₃ could restore in part the functional activity of mutated LXR- α gene found in peripheral blood mononuclear cells of CHD patients. Our data also indicated that CHD patients contain significant low level of vitamin D₃ in their serum and this level is increased in such patients who are treated with atorvastatin. Based upon our findings, it's not unlikely that restoration of serum vitamin D₃ level in such patients may be useful in arresting the progression of coronary atherosclerosis.

Keywords: LXR- α ; CHD patients; Blood mononuclear cells; Vitamin D₃; Withaferin A; +22R (OH) Cholesterol

Introduction

It is alarming to note that 30% of all death that occur world wide are because of cardiovascular disease [1]. There is a general recognition of the fact that co-operativity between lipid peroxidation and inflammation as a result of complex interaction between blood-derived factors, blood mononuclear cells as well as cells within vascular wall lead to coronary heart disease (CHD) [2-4]. Recent observations identify Liver X Receptor (LXR)- α as a molecular link between lipid metabolism and inflammation which are the two pathological hallmarks for the development of CHD [5]. The importance of ligand activated nuclear receptor LXR- α lies in the fact that treatment of atherogenic mouse model with their agonist resulted in significant reduction in lesion development. Pharmacological activation of LXR- α by potent and efficacious ligands, lead to a number of favorable changes in lipid metabolism including promotion of reverse cholesterol transport, inhibition of intestinal cholesterol absorption and the antagonism of inflammatory signaling [6,7]. The athero-protective nature of LXR- α was further strengthened by the studies from our laboratory that not only statins but vitamin 'C' also had the inherent capacity to up regulate genes coding for LXR- α [8]. We also observed significant higher blood cellular LXR- α mRNA expression in subjects belonging to normolipidemic CHD subjects as well as in hyperlipidemic CHD subjects as compared to their corresponding control groups [9]. The selective and conspicuous higher expression of LXR- α gene in CHD patients (with and without hypercholesterolemia) underlines the importance of this gene in the pathogenesis of CHD. Study shows peripheral blood mononuclear cells (PBMCs) expresses the selected 182 candidate cardiovascular gene and thus PBMCs can be exploited as a best model system for the cardiovascular biology system [10].

Our recent study revealed a paradoxical relationship between expression of LXR- α gene versus severity of CHD and existence of deregulated LXR- α transcriptome in peripheral blood mononuclear cells derived from CHD patients. This deregulated LXR- α transcriptome was shown to occur as a result of three critical mutations in the ligand binding domain of LXR- α protein comprising of amino acids Asp324, Pro327 and Arg328, which were responsible for inability of this domain to interact with its natural ligands thereby leading to a impaired ligand receptor interaction which resulted in the deregulation of LXR- α effector

genes [11]. Consequently the present study was directed to explore three specific issues: - a) Does the serum derived from CHD patient contains any factor/(s), which are responsible for the reported genetic aberration in LXR- α gene and deregulation of LXR- α transcriptome? b) Does the serum derived from the normal subjects contain any factor/(s), which can restore the functional aberration of LXR- α gene? c) If yes, what is the nature of such transforming factor/(s)?

Methods

Subject selection and sample collection

Based upon the demographic and laboratory data (age, sex, traditional risk factors and angiographic findings-Table 1), peripheral blood mononuclear cells (PBMCs) and serum was isolated from the 10ml blood collected from the angiographically proven, normolipidemic CHD subjects (age between 18-55 years, N=25), having maximum severity of CHD (Gensini score > 30) [12]. Blood was collected at the 12hour fasting stage and patients were without any medications. PBMCs and serum was isolated from the age matched angiographically proven normolipidemic control subjects (N=25) having normal coronary arteries. PBMCs were isolated by Boyum [13] density gradient centrifugation method using Histopaque (Sigma: solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 \pm 0.001g/ml). Further the blood was immediately processed for the isolation of PBMCs after collection. All the steps were performed at 4°C to avoid RNA degradation. Regarding purity of cells, we found >98% cells were mononuclear and viability count was also found to be >95%.

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Exclusion criteria for both patient and control groups were females, cardiomyopathy, serious organ disease (related to nervous system, respiratory system, digestive system, muscular and urino-genital system), systemic illness (diabetes mellitus, hypertension, AIDS, systemic scleroderma, rheumatoid arthritis, osteoarthritis, systemic lupus erythematosus), chronic alcohol abuse, serious psychiatric illness and anticonvulsant therapy. The investigation conforms to the principles outlined in the declaration of Helsinki [14].

Cell culture experiments: The isolated PBMCs from CHD patients as well as control subjects were maintained in invitro culture medium RPMI-1640 containing antibiotics. The cell viability was assayed by trypan blue dye exclusion method. In order to explore that whether the serum derived from CHD subjects contains any factor which is responsible for the reported genetic aberration in LXR- α gene, PBMCs isolated from normal subject (NC) were exposed with the 10% serum derived from CHD patient (PS). Similarly in order to explore that whether the serum derived from normal subjects contains any factor which can restore the functional aberration of LXR- α gene present in the PBMCs of CHD subjects, PBMCs derived from CHD patient (PC) were exposed with 10% serum derived from normal subject (NS). For both sets of experiments PBMCs derived from normal subjects (NC) exposed with 10% serum derived from the same subject (NS) was taken as basal control. The experiments were performed six times using PBMCs and serum derived from different CHD patients as well as control subjects. Cells exposed to above conditions in triplicate for each subject were incubated at 37°C in CO₂ incubator for 24 hours. These

cells from each culture well were subsequently processed for RNA isolation using standard method [15].

Expression of cellular LXR- α transcriptome: The cDNA was synthesized from equal amount of RNA (1 μ g) derived from each culture well using Revert Aid™ first strand synthesis kit. Based upon previous study with LXR- α knock down cellular model of PBMCs [16], the transcriptional expression of LXR- α gene as well as its effector genes coding for PPAR γ , CD36, LDL receptor, CX3CR1, IL-8, IFN γ was studied using gene specific primers (Table 2) [17-19] and standard RT-PCR method. β actin gene expression was used as invariant control for RNA loading and efficiency of reverse transcription. The PCR amplicons were resolved on ethidium bromide stained 2.5% agarose gels (Figure 1) followed by densitometric scanning of each band using Scion Image Analysis software. Intensity ratio of target mRNA (having amplification pattern of log phase) to β actin mRNA for each gene was expressed as percentage of that in control.

Real time PCR melting curve analysis of LXR- α ligand binding domain: In order to check the LXR- α gene aberration, Ligand binding domain of LXR- α (Forward primer 5' CAGATTGCCCTGCTGAAGAC3' and Reverse primer 5' GAACTCGAAGATGGGGTTGA3') was amplified by real time PCR using 1 \times SYBER GreenER™ qPCR SuperMix Universal, .5 μ M each primer and 1 μ l of cDNA from cDNA synthesis reaction mixture. ROX was used as a reference dye. qPCR reagents were procured from Invitrogen. Further the amplicons were subjected for real time PCR melting curve analysis using temperature ranging

	Control (n =25)	CHD Patient (n=25)	NS= Non Significant NA= Not Applicable
Age	50.7 \pm 6.0	53.0 \pm 8.0	NS
Sex	M	M	NA
TC(mg/dl)	180.4 \pm 6.3	160.3 \pm 8.0	NS
TG(mg/dl)	160.4 \pm 6.2	173.1 \pm 9.4	NS
HDL-C(mg/dl)	47.5 \pm 2.2	42.1 \pm 3.2	NS
LDL-C(mg/dl)	99.6 \pm 8.7	109.0 \pm 7.2	NS
Gensini Score	0.0	34 \pm 5.0	NA
Serum CRP(mg/L)	.75 \pm .26	1.2 \pm .22	NS

Table 1:

S.No.	Gene and PCR product size	Primer Pair	No of PCR Cycles
1	LXR- α (169bp)	Forward: 5'- CAGATTGCCCTGCTGAAGAC -3' Reverse: 5'- GAACTCGAAGATGGGGTTGA -3'	38
2	PPAR γ (474bp)	Forward: 5'- TCTCTCCGTAATGGAAGACC -3' Reverse: 5'- GCATTATGAGACATCCCCAC -3'	38
3	CD36 (389bp)	Forward: 5'- GAGAACTGTTATGGGGCTAT -3' Reverse: 5'- TTCAACTGGAGAGGCCAAAGG -3'	26
4	LDLR (258bp)	Forward: 5'- CAATGTCTCACCAAGCTCTG -3' Reverse: 5'- TCTGTCTCGAGGGGTAGCTG -3'	31
5	CX3CR1 (410bp)	Forward: 5'- CCGAGGTCCTTCAGGAAATCT -3' Reverse: 5'- TCAGCATCAGGTTCAGGAACTC -3'	27
6	IL-8 (300bp)	Forward: 5'-ATGACTTCCAAGCTGGCCGTGGCT -3' Reverse: 5'- TCTCAGCCCTCTTCAAAACTTCT -3'	30
7	IFN γ (423bp)	Forward: 5'- CGTTTTGGGTTCTCTTGGCTGTT -3' Reverse: 5'- CTCCTTTTTCGCTTCCCTGTTTT -3'	35
8	β actin (256 bp)	Forward: 5'- TCCTTAATGTCACGCACGAT -3' Reverse: 5'- CATGTAGGTTGCTACCAGGC -3'	20

Table 2:

from 50°C to 95°C. Confirmation of melting curve analysis was done by direct sequencing of the amplicons using an automated ABI 3100 Genetic Analyzer (Bangalore Genei) using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry (Sanger's Sequencing method).

In silico search for analogue of mutated LXR- α ligands: Based upon the homology modeling we have already reported a distortion in the ligand binding cavity of LXR- α derived from CHD subjects, as well as by docking experiments we have shown that due to altered structure, synthetic and natural LXR- α agonist are unable to activate the LXR- α protein [11]. As the binding of natural and synthetic ligand to the LXR- α ligand binding domain are mediated by interaction of hydroxyl groups of ligand with Trp443 and His421 [20], we wanted to search the compound which could interact with Trp and His in the ligand binding cavity of LXR- α protein derived from CHD patients. For this we scanned several intermediates of cholesterol biosynthesis pathway as well as cholesterol derivatives (7- α hydroxy cholesterol, squalene, lanosterol, prenenole, progesterone, aldosterone, testosterone, estradiol, cortisol, corticosterone and vitamin D₃) using AutoDock 4.0. Structure of cholesterol derivatives were modeled by sketch tool of Discovery Studio 2.0, accelrys. The complex of LXR- α ligand binding domain (derived with normal and CHD subjects) with cholesterol derivative compounds were built by AutoDock 4.0 [21]. This docking procedure was implemented as a Monte carlo (MC) simulated annealing exploration with a rapid evaluation using grid-based molecular affinity potentials. We have specified a rectangular volume around the LXR- α ligand binding cavity and the cholesterol derivative compounds were assumed to be flexible that have resulted 10 best conformations according to the free energy of binding. We have chosen the conformation having maximum free energy of binding. The molecular diagrams of the complexes were drawn by Pymol software [22].

Functional activity of LXR- α in response to vitamin D₃, +22R (OH) cholesterol and Withaferin A in patient cells: To check the functional activity of mutated LXR- α in response to vitamin D₃, peripheral blood mononuclear cells were isolated [13] from CHD patients and these cells were exposed with vitamin D₃ (0-50ng/ml) for 24 hours under invitro culture condition on RPMI-1640 medium at 37°C in CO₂ incubator. The functional activity of LXR- α was checked by the expression of its direct target genes coding for LDL receptor and IFN γ [23,24]. Further the LXR- α activity was also checked by exposing the peripheral blood mononuclear cells derived from CHD subjects to its natural ligand +22R hydroxyl cholesterol (10 μ M) and Withaferin A (10 μ M).

Effect of vitamin D₃ on LXR- α knock out patients PBMCs: LXR- α siRNA as well as scrambled siRNA (control) (Santa Cruz Biotechnology, USA) were transfected into PBMCs derived from CHD,

using transfection reagent (Santa Cruz Biotechnology, USA). In order to access efficiency of siRNA transfection flourescein-A conjugated control scrambled sequence was employed. The efficiency was found to be about 70-80%. After 48 hours of transfection vitamin D₃ (50ng/ml) was added to siRNA treated patient cells as well as siRNA untreated cells. After 72 hours expression of LXR- α and its target genes i.e. LDLR [23] and IFN γ [24] were analyzed.

Reporter activity of LXR- α response element

To further confirm the activity of LXR- α protein derived from normal and CHD subjects in response to its ligands, reporter assay was performed using TOPO[®] Reporter Kit (Invitrogen). LXR- α response element was amplified by PCR and was incorporated into the pBlue TOPO[®] TA cloning vector by the manufacturer protocol. This vector containing the LXR- α response element was transfected to the cultured peripheral blood mononuclear cells derived from normal and CHD subjects (on antibiotic free and serum free RPMI-1640 medium) by siPORT[™] XP-1 transfection agent (Applied Biosystems). After 6 hours of transfection cells were exposed to +22R hydroxyl cholesterol (10 μ M), Withaferin A (10 μ M) and Vitamin D₃ (50ng/ml) for 72 hrs. β -galactosidase activity was measure by β -Gal Assay Kit (Invitrogen). (Control was taken as cells transfected with vector without exposing to any ligand).

Measurement of 25(OH) vitamin D₃: Serum vitamin D₃ level in normal (n=25), untreated CHD patient (n=25) and in the atorvastatin treated CHD patients (20mg/day oral administration for more than 6 months) (n=25) were measured by Immuno chemiluminescence Analyzer (Roche Hitachi, Elecsys 2010) using commercial kit (Roche).

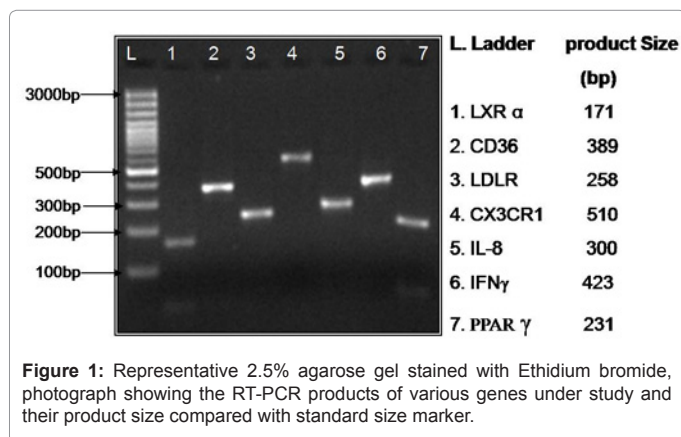
Statistical Analysis: Statistical Analyses were performed with the use of SPSS Windows version 13. All the data have been expressed as meanSD. Students t-test was used to compare the groups. P values less than 0.05 were taken to be significant.

Results

Expressional studies of LXR- α and its effector genes: Exposure of peripheral blood mononuclear cells from the control group to serum derived from CHD group (NC+PS) resulted in significant increased transcriptional expression of genes coding for LXR- α , PPAR γ , CD36 and LDL receptor which was accompanied by significant decreased expression of genes coding for CX3CR1, IL-8 and IFN γ as compare to corresponding control (NC+NS) (Figure 2). Exposure of cells derived from patient to serum derived from normal subjects (PC+NS) resulted in significant transcriptional over expression of genes coding for LXR- α , PPAR γ and CD36 which was accompanied by no significant change in the expression of genes coding for LDL receptor, CX3CR1, IL-8 and IFN γ as compare to corresponding control (NC+NS) (Figure 3). Taken together these results revealed PBMC's derived from normal subject's exhibit a well regulated LXR- α transcriptome that is not affected by the exposure of serum derived from CHD patients. Further PBMC's derived from patient's contain a deregulated LXR- α transcriptome which cannot be restored by the serum derived from normal subjects.

Real time PCR melting curve analysis of LXR- α ligand binding domain

Study of real time PCR melting curve analysis revealed that melting pattern of LXR- α ligand binding domain from normal cells was not altered by the exposure of normal cells with patient serum (NC+PS) as compared to normal cells exposed with normal serum (NC+NS) (Figure 4A). Further a differential melting pattern of LXR- α ligand binding domain of the patient cells exposed with normal



serum (PC+NS) was found as compared to normal cells exposed with normal serum (NC+NS) (Figure 4B). Sequencing of the amplicons also confirmed an intrinsic genetic aberration independent of serum factors. Taken together melting curve study revealed that patient serum does not contains any factor which is responsible for the cellular LXR- α gene aberration, further serum derived from normal subjects does not contains any factor which can restore the previously reported intrinsic genetic aberration in LXR- α ligand binding domain [11].

In silico search for analogous of LXR- α ligands

By the *in vitro* cell culture experiment we found serum derived from normal subjects does not contain any factor that can restore the

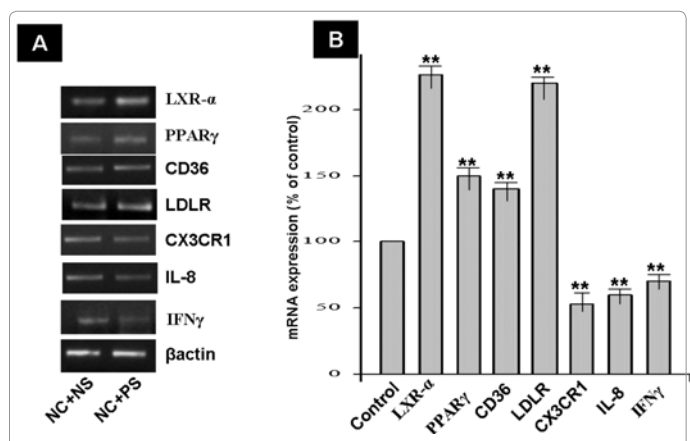


Figure 2: A) Representative agarose gel photographs showing ethidium bromide stained RT-PCR products in Normal cells exposed with normal serum (NC+NS) and normal cells exposed with patient serum (NC+PS). B) The signal intensities of these RT-PCR products were measured using SCION IMAGE analysis software. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β actin band intensity. The mRNA expression of each gene in NC+PS is plotted as percentage of that in NC+NS. Each bar represents mean \pm SD for the combined results of six separate experiments from different individuals in triplicate. (Statistical significance is shown by ** = $p < 0.05$).

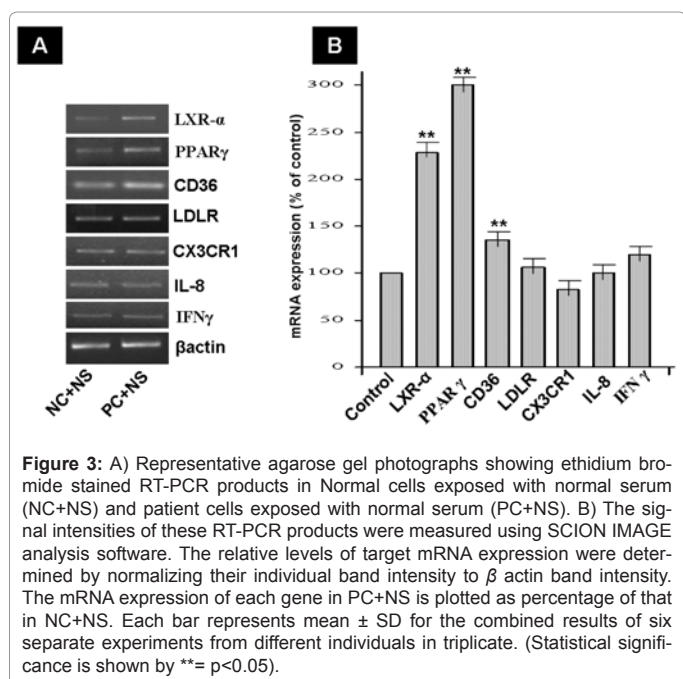


Figure 3: A) Representative agarose gel photographs showing ethidium bromide stained RT-PCR products in Normal cells exposed with normal serum (NC+NS) and patient cells exposed with normal serum (PC+NS). B) The signal intensities of these RT-PCR products were measured using SCION IMAGE analysis software. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β actin band intensity. The mRNA expression of each gene in PC+NS is plotted as percentage of that in NC+NS. Each bar represents mean \pm SD for the combined results of six separate experiments from different individuals in triplicate. (Statistical significance is shown by ** = $p < 0.05$).

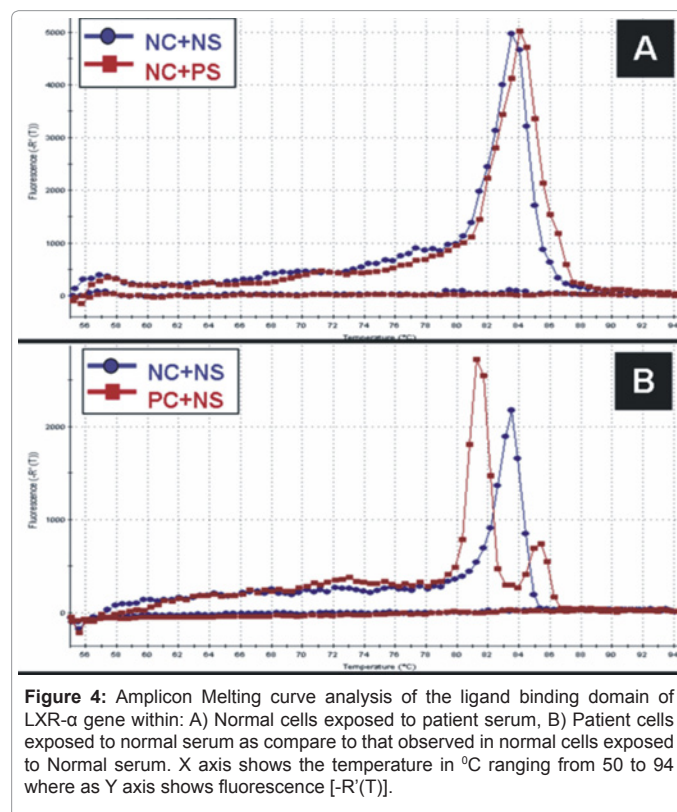


Figure 4: Amplicon Melting curve analysis of the ligand binding domain of LXR- α gene within: A) Normal cells exposed to patient serum, B) Patient cells exposed to normal serum as compare to that observed in normal cells exposed to Normal serum. X axis shows the temperature in $^{\circ}\text{C}$ ranging from 50 to 94 where as Y axis shows fluorescence [-R'(T)].

functional aberration of LXR- α gene reported previously [11]. So we wanted to search the analogue of LXR- α ligand which can interact with Trp and His [20] residue present in the ligand binding cavity of mutated LXR- α protein. By the docking experiments, out of several intermediates of cholesterol biosynthesis pathway and cholesterol derivative compounds, the binding affinity of LXR- α ligand binding domain with vitamin D_3 was found to be most satisfactory. In vitamin D_3 only one hydroxyl group is present at position-1. We have found that there is no hydrogen bond between vitamin D_3 and Trp443 or His421 residue of normal LXR- α (Figure 5A). However, in vitamin D_3 -mutated LXR- α ligand binding domain complex, the methyl group is positioned on top of His residue facing the π -electron cloud of imidazole ring (Figure 5B). The distance between the methyl-carbon and imidazole nitrogen is 4.2 Å. There are reports that hydrogen bonds with π -acceptors through C-H... π interaction can stabilize proteins [24-26]. So here this orientation of the residues may facilitate hydrogen bond between vitamin D_3 and His amino acid residue which is not present in normal LXR- α ligand binding domain-vitamin D_3 complex. Using ICM software (version 2.8; Molsoft Inc.) [27] Svensson et al. docked synthetic and naturally occurring ligands with normal LXR- α ligand binding domain [20]. In our previous paper we reported the same results using AUTO DOCK [11]. Here also the ligand binding residue His is supplementing the binding interaction between vitamin D_3 and mutated LXR- α ligand binding domain. Further we have also reported the lesser free energy as calculated by AutoDock' own procedure for the ranked best complex of normal LXR- α Ligand binding domain with vitamin D_3 as compare to complexes of vitamin D_3 with mutant LXR- α Ligand binding domain i.e. $-10.65 \text{ kcal mol}^{-1}$ and $-10.74 \text{ kcal mol}^{-1}$ respectively. It is needless to mention here that this free energy of binding is in the similar range as we obtained in our previous oxysterol-LXR- α ligand binding domain complex [11]. Taken together docking result suggest that the LXR- α protein derived from CHD subjects can be activated by vitamin D_3 .

Functional activity of LXR- α in response to vitamin D₃, +22R (OH) cholesterol and Withaferin A in patient cells

To further confirm the activation of LXR- α protein derived from CHD patient by vitamin D₃ PBMC's derived from CHD subjects were exposed with vitamin D₃ (0-50 ng/ml) under invitro cell culture for 24 hours and the expression of LXR- α and its direct target genes coding for LDLR and IFN γ were studied [23,24]. We found a significant increase in the expression of LDLR and decrease in the expression of IFN γ at 30ng/ml (Figure 6). Further no significant change was found in the expression of LXR- α , LDLR and IFN γ after exposure of PBMC's derived from CHD subjects with +22R(OH) cholesterol and Withaferin A (Figure 7). Taken together these results suggest the activation of mutated LXR- α , observed in the blood mononuclear cells of CHD patients by vitamin D₃ rather than its natural ligand that is +22R(OH) cholesterol.

Effect of vitamin D₃ on LXR- α knock down patients PBMCs

To further check that effect of vitamin D₃ are through mutant LXR- α , we performed the LXR- α silencing in PBMCs derived from CHD subjects and exposure of these cells with vitamin D₃ does not altered the expression of LDLR and IFN γ significantly, where as exposure of LXR- α siRNA untransfected cells with vitamin D₃ altered the expression LDLR and IFN γ (Figure 8).

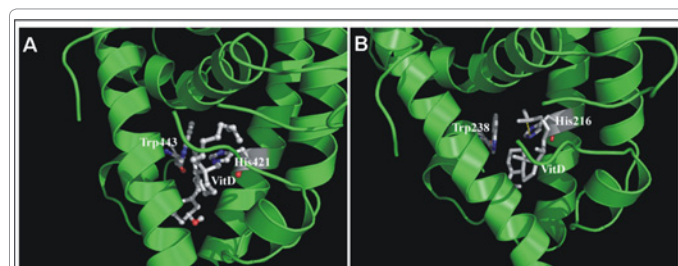


Figure 5: Ribbon diagrams with the orientations of vitamin D₃ and hydroxyl group interacting tryptophan and histidine residues (Trp443 and His421 in normal LXR- α protein (A) while Trp238 and His216 in patient LXR- α protein (B) in ball-and-stick mode at the ligand binding cavities are shown. The hydrogen bond between vitamin D₃ and histidine residue of the ligand binding domain is shown by yellow color dotted line. In each figure the tryptophan and histidine residues are labeled by three-letter amino acid codes followed by the residue numbers. The vitamin D₃ is labeled as vitD.

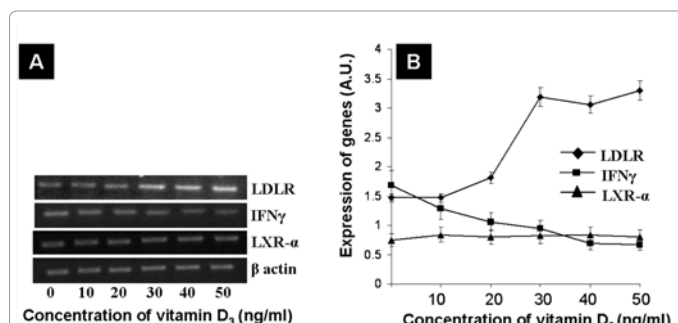


Figure 6: Dose dependent effect of vitamin D₃ on the expression of LXR- α , IFN γ and LDLR in PBMC's derived from CHD patient. A) Representative agarose gel photographs showing ethidium bromide stained RT-PCR products. B) The signal intensities of these RT-PCR products were measured using SCION IMAGE analysis software. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β actin band intensity. Each bar represents mean \pm SD for the combined results of three separate experiments. (Statistical significance is shown by **= p<0.05).

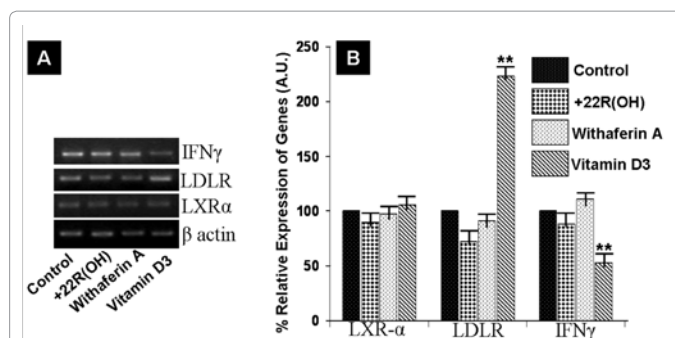


Figure 7: Expression of LXR- α , IFN γ and LDLR after exposure of PBMC's derived from CHD patient with various ligands. +22R(OH)=10 μ M, Withaferin A=10 μ M, Vitamin D₃=50ng/ml. A) Representative agarose gel photographs showing ethidium bromide stained RT-PCR products. B) The signal intensities of these RT-PCR products were measured using SCION IMAGE analysis software. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β actin band intensity. Each bar represents mean \pm SD for the combined results of three separate experiments. (Statistical significance is shown by **= p<0.05).

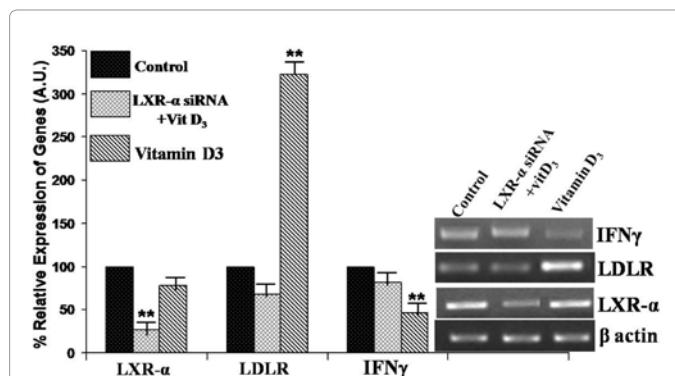


Figure 8: Effect of vitamin D₃ (50ng/ml) on LXR- α knock out PBMCs derived from CHD patients. Control is the patient PBMCs transfected with scrambled siRNA sequence. Right panel shows representative agarose gel photographs showing ethidium bromide stained RT-PCR products and the left panel shows signal intensities of these RT-PCR products using SCION IMAGE analysis software. The relative levels of target mRNA expression were determined by normalizing their individual band intensity to β actin band intensity. Each bar represents mean \pm SD for the combined results of three separate experiments. (Statistical significance is shown by **= p<0.05).

Reporter activity of LXR- α response element

In order to confirm that vitamin D₃ is exerting its effect through LXR- α , we performed a transient transfection assay with a reporter plasmid containing LXR- α response element in PBMC's derived from both normal and CHD subjects. In the cells derived from the normal subjects we found a significant increase in the LXR- α response-element dependent β galactosidase activity in response to +22R (OH) cholesterol and Withaferin A, whereas we did not find the significant change in response to vitamin D₃. Further in the cells derived from CHD subjects, we found a significant increase in the LXR- α response element dependent β galactosidase activity in response to vitamin D₃, whereas we did not find significant change in response to +22R (OH) cholesterol and Withaferin A (Figure 9).

25(OH) vitamin D₃ level in serum

Keeping in view these results that vitamin D₃ is exerting its effect through LXR- α in CHD patient as well as has the potential to restore the functional activity of mutated LXR- α , it became imperative to check

the serum level of vitamin D₃ in normal and CHD subjects. Further statins are the drug of choice for the treatment of CHD [28,29], we were interested to check the vitamin D₃ level of statin treated CHD patients also. By immuno-chemiluminescence analyzer we not only found a significant decreased level of serum 25(OH) vitamin D₃ level in CHD patient group as compare to normal group but also found a significant increased level of vitamin D₃ in the serum of atorvastatin treated CHD patient group as compare to corresponding control group (Figure 10).

Discussion

There exists a general recognition of the fact that LXR- α acts as a crucial molecular switch for initiating a cross talk between lipid peroxidation and inflammation observed in atherogenesis [2-5]. Although LXR- α agonist have been shown to have cardio protective role in experimental atherosclerotic animal model systems [6,7], our recent study revealed a paradoxical relationship between LXR- α gene expression within peripheral blood mononuclear cells derived from

CHD patients and the severity of CHD in such patients [11]. This apparent paradox could be resolved by the finding that revealed three critical mutations in the ligand binding domain of LXR- α comprising of amino acids Asp324, Pro327 and Arg328, thereby making the ligand binding domain of this protein unable to interact with known natural ligands of LXR- α [11]. This finding prompted us to explore whether or not the observed mutated LXR- α gene in CHD patients was a result of intrinsic genetic aberration or some abnormal serum factors in such subjects.

The results reported here unambiguously attest to the fact that the serum of CHD patients does not contain any factor that had the capacity to induce deregulated blood cellular LXR- α transcriptome in such patients. Consequently based upon these and our previous results, it is reasonably to assume that there exists an intrinsic genetic defect in LXR- α gene within blood cells of CHD patients [11]. Further an attempt was made to screen various molecules (having analogous structure to natural LXR- α agonists) that had the inherent capacity to activate mutated LXR- α protein and restore its functionality. Our molecular docking results depicted that vitamin D₃ could activate mutated LXR- α protein by interaction with His amino acid (Figure 5B) and stabilizing through hydrogen bonds with π -acceptors through C-H... π interaction [24-26]. This observation was further confirmed unambiguously by our experiments (Figure 6-9) showing that mutated LXR- α protein derived from PBMCs of CHD patients could be activated through exposure to vitamin D₃ *in vitro* culture of these cells resulting in regulation of LXR- α effector genes. These findings prompted us to measure the serum vitamin D₃ level in CHD patients as compare to their matched controls. It was interesting to note that serum derived from CHD patients did contain significant lower levels of vitamin D₃ as compared to their corresponding controls (Figure 10), which is known to be associated with the higher risk of CHD [30-32]. It is pertinent to note at this stage that atorvastatin (recognised widely as a drug of choice in CHD patients across the globe) has been shown to increase the expression of LXR- α mRNA as well as serum vitamin D₃ levels in patients suffering from CHD (Figure 10) [33-35].

Based upon these findings it is not unlikely that correction of vitamin D₃ deficiency observed in CHD patients may have a beneficial effect on the regulation of their mutated LXR- α protein.

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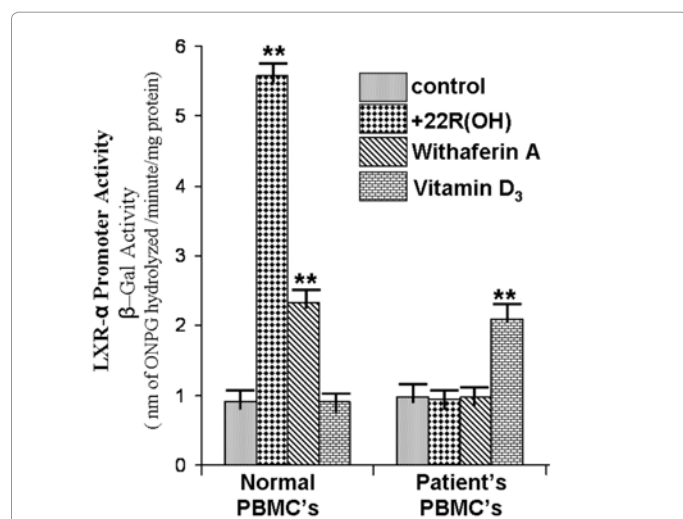


Figure 9: LXR- α promoter dependent β -galactosidase reporter activity in PBMCs derived from normal and CHD patients exposed to different ligands. +22R(OH)=10 μ M, Withaferin A=10 μ M, Vitamin D₃=50ng/ml. Each bar represents mean \pm SD of the experiments done in triplicate. (Statistical significance is shown by **= p<0.05).

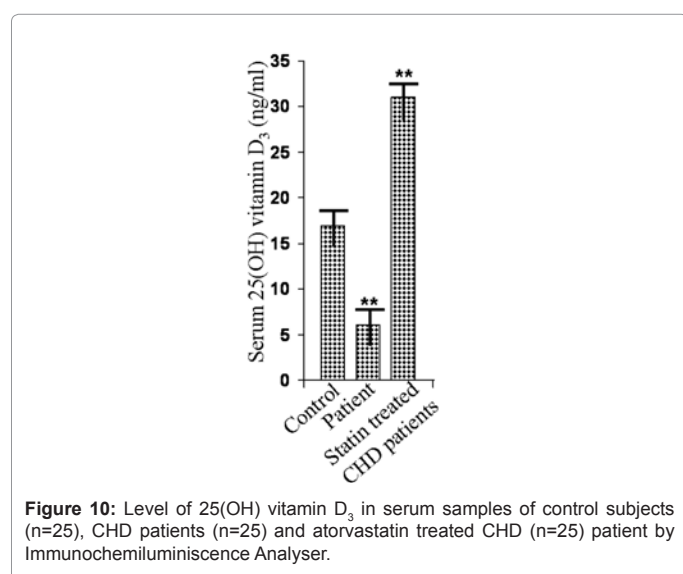


Figure 10: Level of 25(OH) vitamin D₃ in serum samples of control subjects (n=25), CHD patients (n=25) and atorvastatin treated CHD (n=25) patient by Immunochemiluminiscence Analyser.

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